Targeting Receptor Tyrosine Kinase Signaling in Acute Myeloid Leukemia

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ABBREVIATIONS

AML	Acute Myeloid Leukemia
Ara-C	Cytarabine
ATRA	All-*Trans*-Retinoic Acid
AT/RT	Atypical Teratoid/Rhabdoid Tumor
CBF	Core Binding Factor
CBF-AML	Core Binding Factor Leukemia
CBP	Creb-Binding Protein
CML	Chronic Myeloid Leukemia
CNS	Central Nervous System
CSF-I	Colony-Stimulating Growth Factor I
4E-BP	4E-Binding Protein
EGF	Epidermal Growth Factor
ERK	Extracellular Signal-Regulated Kinase
FAB	French-American-British Classification
FGF	Fibroblast Growth Factor
FKHR	Forkhead
FLT3	Fms-like Tyrosine Kinase 3
FL	FLT3 Ligand
FTI	Farnesyltransferase Inhibitor
GBM	Glioblastoma Multiforme
GPCR	G-Protein Coupled Receptor
GSK-3	Glycogen Synthase Kinase-3
IGF	Insulin-like Growth Factor
IGFBP1-6	Insulin-like Growth Factor Binding Protein 1-6
IGF-IR	Insulin-like Growth Factor I Receptor
IR	Insulin Receptor
IRS	Insulin Receptor Substrates
ITD	Internal Tandem Duplication
JAK	Janus Protein Tyrosine Kinase
M-6-PR	Mannose 6-Phosphate Receptor; IGF-II Receptor
MB	Medulloblastoma
MDM-2	Murine Double Minute Gene 2

MDS	Myelodysplastic Syndrome
MM	Multiple Myeloma
MPD	Myeloproliferative Disease
MRT	Malignant Rhabdoid Tumor
mTOR	Mammalian Target of Rapamycin
NB	Neuroblastoma
PDGF	Platelet-Derived Growth Factor
PDK1	Phosphoinositide-dependent Kinase-1
PI	Phosphatidylinositol
PI3K	Phosphoinositide 3-Kinase
PI(3)P	Phosphatidylinositol-3-Monophosphate
$PI(3,4)P_2$	Phosphatidylinositol-3,4-Bisphosphate
$PI(3,4,5)P_3$; PIP_3	Phosphatidylinositol-3,4,5-Trisphosphate
PKB	Protein Kinase B (also termed Akt)
PTK	Protein Tyrosine Kinase
RA	Rheumatoid Arthritis
RARα	Retinoic Acid Receptor Alpha
Rheb	Ras Homologue Enriched in the Brain
RNAi	RNA Interference
RTK	Receptor Tyrosine Kinase
S6K	Ribosomal Protein S6 Kinase
SCLC	Small Cell Lung Cancer
Shc	Src-Homology Collagen Protein
shRNA	Small Hairpin RNA
SOS	Son of Sevenless
STAT	Signal Transducer and Activator of Transcription
TSC1, TSC2	Tuberous Sclerosis Complex 1 and 2

1. A. SUMMARY

The acute myeloid leukemia (AML) is a quickly progressing, heterogeneous clonal disorder of hematopoietic progenitor cells. A significant progress in the understanding the pathogenesis of AML has been achieved in the past few years. Two major types of genetic events are thought to give rise to leukemic transformation: alterations in the activity of transcription factors controlling hematopoietic differentiation and activation of components of receptor tyrosine kinase (RTK) signaling pathways. This has led to the development of promising new therapeutic strategies for the disease. Recent developments in the field of molecularly targeted therapies for AML involve RTKs such as the FMS-like tyrosine kinase 3 (Flt3), c-Kit and signal transduction via the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. Initial results imply that targeting RTKs is a very promising approach for AML and that other receptors, such as the insulin-like growth factor receptor (IGF-IR), could also represent new targets in the future.

The main goal of this work was therefore aimed at gaining deeper insight into RTK signaling in a panel of AML cell lines and patient blast cells. A broad potein expression analysis was set up in order to identify cancer-specific expression patterns of signaling molecules and to uncover potential molecular targets. Using different approaches the molecular function and the feasibility of targeting the candidate proteins was investigated. Novel specific inhibitors were tested and neutralizing antibodies and RNA interference (RNAi) were used to block or down-regulate the individual proteins. Our study of the IGF-IR/PI3K signaling system in AML cells uncovered a novel role for autocrine IGF-I signaling in the growth and survival of these cancer cells. Besides, we could show that targeting this signaling branch in combination with commonly used chemotherapeutical agents represents an interesting novel approach for AML therapy.

As protein expression analysis revealed highly variable expression levels of the mammalian target of rapamycin (mTOR) in the panel of cells analyzed, further interest was laid on the role of mTOR in AML. Cells expressing low levels of mTOR were compared to cells expressing high levels of this protein and a siRNA screen was aimed at uncovering human kinases that modulate the sensitivity to the mTOR inhibitor rapamycin. Preliminary results suggest that mTOR plays a crucial role in cell growth and survival in a subset of AML cells and that rapamycin in combination with certain RTK or Syk/ZAP70 inhibitors has potential for AML therapy.

Another study investigating RTK signaling in childhood central nervous system (CNS) tumors revealed autocrine insulin production by the cancer cells and a crucial role of this growth factor and the insulin receptor (IR)/PI3K signaling pathway for cellular growth and survival of the malignant cells.

Moreover, our interest was set on elucidating the specific role of individual PI3K isoforms in human cancer. The PI3Ks are a family of lipid kinases which transduce signals from RTKs and play a crucial role in a variety of intracellular signaling events including the control of cell growth and survival. Nevertheless, the specific functions of the individual isoforms in human cancer has not been completely understood yet. In view of the search for specific molecular targets for cancer therapy, an extensive knowledge of the exact role and function of these signaling enzymes is of high importance. Therefore, PI3K isoforms were specifically targeted in human cancer cells and the effect on cellular responses was investigated. In AML, a predominant role of the class I_A PI3Ks p110β and p110δ for cell growth, survival and chemo-resistence could be described. In an extensive study of PI3K isoforms in human neuroblastoma, a novel function of p110δ in growth and survival of this type of cancer could be identified. Since much less attention has been paid to the role of the class II PI3Ks in human cancer, our work was aimed at elucidating the expression pattern and function of PI3KC2β in a panel of human tumor cells including AML, different brain tumors and neuroendocrine tumors. Novel isoform-specific inhibitors were tested and with the use of RNAi it could be shown that PI3KC2β contributes to cell survival, proliferation and migration of various human cancer cells.

In summary, our study of the RTK/PI3K signaling system in distinct human cancers has provided novel insights into the importance and the complexity of this network for tumor growth and survival. Moreover, the analysis of specific components of this crucial signaling network has uncovered potential molecular targets for future cancer therapy.

1. B. ZUSAMMENFASSUNG

Die akute myeloide Leukämie (AML) ist eine bösartige monoklonale Krankheit der hämatopoitischen Vorläuferzellen mit einem rasch progredienten klinischen Verlauf. Hinsichtlich der Ätiopathogenese konnten aber in den letzen Jahren wichtige neue Erkenntnisse gewonnen werden, so so dass man heute v.a. von zwei essentiellen genetischen Veränderungen ausgeht, die hauptsächlich zur Entstehung dieser Krebsart beitragen: 1) Veränderungen in der Aktivität von Transkriptionsfaktoren, welche die Differenzierung der hämatopoitischen Zellen kontrollieren, sowie 2) die Aktivierung von Komponenten des Rezeptor-Tyrosinkinase (RTK)-Signalweges. Diese Faktoren stellen eine wichtige Grundlage für die Entwicklung neuartiger Strategien in der Krebstherapie dar. Aktuelle Errungenschaften im Gebiet der zielgerichteten AML-Therapien fokusieren bereits auf RTKs. Zudem gibt es Studien, welche zum Beispiel die FMS-like tyrosine kinase 3 (FLT3), c-Kit oder die Signalübermittlung via phosphoinositide 3-kinase (PI3K) und die mitogen-activated protein Kinase (MAPK) als molekulares Ziel beschreiben. Erste Resultate zeigen auch, dass die RTKs als therapeutisches Ziel eine sehr erfolgversprechende Strategie darstellen. Eine Vielzahl weiterer Kandidaten aus dieser Gruppe - wie zum Beispiel der insulin-like growth factor receptor (IGF-IR) - wird gegenwärtig in vitro sowie in präklinischen Studien untersucht .

Der Hauptfokus meiner Doktorarbeit lag daher im detaillierten Studium der Signalübermittlung durch RTKs in verschiedenen AML-Zelllinien und Vorläuferzellen (Blasten) von AML-Patienten. Eine ausführliche Analyse galt dabei der Expression von verschiedenen Proteinen, welche eine Rolle in der intrazellulären Signalübertragung spielen, um so ein ein spezifisches Musters für Krebszellen zu identifizieren. Mit Hilfe von verschiedenen Techniken wurde dann die genaue molekulare Funktion der Kandidaten untersucht und ihr Potential als therapeutisches Ziel analysiert. Schliesslich wurden neue, spezifische Inhibitoren getestet und neutralisierende Antikörper und RNA-Interferenz (RNAi) zur zielgerichteten Hemmung der gewünschten Proteine angewendet.

Eine umfangreiche Analyse des IGF-IR/PI3K Netzwerkes in AML-Zellen zeigte insbesondere, dass die leukämischen Zellen in der Lage sind, IGF-I selber zu produzieren und zu sezernieren. Damit konnte eine neue, autokrine Rolle von IGF-I für das Wachstum und Überleben der Krebszellen beschrieben werden. Zugleich konnten wir zeigen, dass dieses System ein

vielversprechender molekularer Angriffspunkt in der Krebstherapie ausmacht, v.a. in Kombination mit klinisch bereits eingesetzten Chemotherapeutika.
Daneben zeigte die Analyse der Proteinexpression in AML-Zellen eine ausgeprägte Variabilität des mammalian target of rapamycin (mTOR). In einem nächsten Schritt haben wir deshalb die funktionelle Rolle von mTOR in AML weiter untersucht. Dabei wurden Zelltypen mit einer starken Expression von mTOR gegenüber einer geringen mTOR-Expression verglichen. Mittels eines siRNA-screens suchten wir zudem nach Kinasen, welche die Sensitivität zum mTOR Inhibitor Rapamycin beeinflussen. Erste Resultate zeigten, dass mTOR eine wichtige Rolle für das Wachstum und Überleben in einer Gruppe von AML Zellen spielt und dass Rapamycin in Kombination mit gewissen RTK oder SYK/ZAP70 Inhibitoren als mögliche neue Therapeutika der AML in Frage kommen.

In einer anderen Studie konnten wir zeigen, dass in pädiatrischen Tumoren des Zentralnervensystems (ZNS) die autokrine Produktion von Insulin eine essentielle Rolle für das Wachstum und Überleben der Krebszellen spielt.

Des Weiteren wurde die spezifische Funktion der verschiedenen PI3K Isoformen in Krebs untersucht. Die PI3Ks bilden eine Familie von Kinasen, welche als wichtige Mediatoren von intrazellulären Signalen eine Vielfalt von zellbiologischen Vorgängen kontrollieren, in erster Linie aber Wachstum und Überleben der Zelle. Dennoch fehlen bis heute weitgehend Studien, welche die verschiedenen PI3K-Isoformen nach ihrer spezifischen Relevanz untersucht haben. Die Suche nach geeigneten molekularen Zielen für die Krebstherapie stellt jedoch unumgänglich eine fundierte Kenntnis der exakten Funktion von potentiellen Kandidaten dar. Daher haben wir spezifische PI3K-Isoformen untersucht und ihre Rolle in zellulären Antworten analysiert. In AML konnten so die PI3K-Isoformen p110δ und p110β als wichtigste Isoformen für Wachstum und Überleben der Krebszelle sowie deren Resistenz gegenüber Chemotherapeutika identfiziert werden. Diese Daten konnten zudem in einer Analyse von PI3K-Isoformen in Neuroblastomen bestätigt werden. Da der Rolle von PI3Ks der Klasse II in Krebszellen bis heute noch kaum Beachtung geschenkt wurde, haben wir eine umfangreiche Studie zur Expression und Funktion von PI3KC2β in verschiedenen Krebsarten (AML, Hirntumoren und neuroendokrine Tumoren) durchgeführt. Es wurden neue Isoform-spezifische Inhibitoren getestet und mittels RNAi konnte

gezeigt werden, dass PI3KC2β zum Überleben und Wachstum, wie auch zur Migration von Krebszellen beiträgt.

Zusammengefasst gibt die vorliegende Dissertation bedeutende Einblicke in die Komplexität des RTK/PI3K Netzwerkes und unterstreicht die Wichtigkeit dieses Signalsystemes für das Wachstum und Überleben von Krebszellen. Zudem konnten durch die fundierte Untersuchung von einzelnen Komponenten dieses Systemes neue potentielle Ziele zur Krebstheapie identifiziert werden.

2. INTRODUCTION

2.1. Acute Myeloid Leukemia

Acute leukemias represent a small (<3%) fraction of all human cancers, but are a leading cause of cancer-related mortality in children and young adults (Deschler & Lubbert, 2006; Kolitz, 2006). The acute myeloid leukemia (AML) accounts for about 25% of all leukemias in adults in the Western world, and is the second most frequent form of leukemia following chronic lymphocytic leukemia (CLL) (Deschler & Lubbert, 2006). The incidence of AML is 3.4 cases per 100,000 general population. AML is a heterogeneous group of diseases characterized by uncontrolled clonal proliferation of hematopoietic precursor cells leading to impairment of normal hematopoietic cell production, causing cytopenias. Based on the cell type of which the leukemia cell developed together with the degree of its maturity, the French-American-British (FAB) classification system groups AML into eight subtypes, M0 through M7 (Table 1). The different subtypes are known to have varying clinical prognoses and therapy responses.

French-American-British Classification (FAB)	
M0	*undifferentiated AML* the bone marrow cells show no significant signs of differentiation
M1	*myeloblastic leukemia, without differentiation* the bone marrow cells show minimal signs of maturation (granulocyte differentiation)
M2	*myeloblastic leukemia, with differentiation* the bone marrow cells are at or beyond the promyelocyte stage (early granulocyte)
M3	*promyelocytic leukemia* most cells are abnormal early granulocytes between the stage of myeloblasts and myelocytes
M4	*myelomonocytic leukemia* variable amounts of differentiated granulocytes and monocytes in the bone marrow and circulating blood; > 20% monocytes and promonocytes in the bone marrow
M5	*monoblastic or monocytic leukemia* poorly differentiated monoblasts (immature monocytes) with lacy-appearing genetic material or large population of monoblasts, promonocytes, and monocytes
M6	*erythroleukemia* abnormal red blood cell-forming cells making up over half of the nucleated cells in the bone marrow
M7	*megakaryoblastic leukemia* immature megakaryocytes or lymphoblasts

Table 1: French-American-British Classification of AML

The World Health Organization Classification (WHO) subdivides the AMLs into 4 major categories: AML with recurrent cytogenetic translocations; AML with multilineage dysplasia; AML and myelodysplastic syndromes (MDS), therapy-related; and AML not otherwise categorized (Table 2). This system provides prognostic information in terms of cytgenetic findings and associated myelodysplastic changes in the AML.

World Health Organization Classification (WHO)	
AML with recurrent cytogenetic translocations	t(8;21)(q22;q22) AML1/CBFα/ETO t(15;17)(q22;q12) and variants PML/RARα inv(16)(p13;q22) t(16;16)(p13;q22) CBFβ/MYH1 11q23 MLL abnormalities Patients generally have a high rate of remission and a better prognosis compared to other types of AML.
AML with multilineage dysplasia	Myelodysplastic syndrome (MDS) or myeloproliferative disease (MPD) that transformed into AML. Occurs often in elderly patients and often with a worse prognosis.
AML and MDS, therapy-related	AML or MDS developed after chemotherapy and/or radiation therapy. Often characterized by specific chromosomal abnormalities and often with a worse prognosis.
AML not otherwise categorized	AML subtypes that do not fall into the above categories.

Table 2: World Health Organization Classification of AML

During the past decade, there has been a shift in AML diagnosis, classification, and treatment strategies from protocols relying on cell morphology and cytochemistry, to cell-surface antigen expression and the use of cytogenetic and molecular markers. Treatment of AML, however, still represents a difficult challenge because of relapse and complications associated with treatment (Kolitz, 2006). In the past 40 years, advances in supportive care and development of chemotherapeutic agents have led to improved outcomes in patients with acute myeloid leukemia. High relapse rates following remission have led to extensive efforts to develop techniques and regimens for detecting and eliminating minimal residual disease. Since standard cytotoxic chemotherapies have side effects to which AML patients, especially those of advanced age, are susceptible, there is a need to develop new therapies to overcome these difficulties (Deschler & Lubbert, 2006; Estey & Dohner, 2006). A better understanding of the biology and the molecular pathogenesis of AML has led to the development of new, targeted agents and strategies for AML treatment. Targeted therapy has indeed improved outcomes in some AML patients. Most of the

new agents used for AML therapy are less toxic then their predecessors, and combinations with more intensive traditional regimens are being tested.

2.1.1. Pathogenesis

Great efforts have been made to gain a better understanding of the molecular pathogenesis of AML. The malignancy is characterized by an accumulation of granulocyte or monocyte precursors in the blood. This is caused by the clonal growth of immature progenitor cells, which display increased proliferation and resistance to apoptosis, as well as impaired differentiation (Steffen et al., 2005).

Recurring chromosomal translocations are a hallmark of human leukemias. There are more than 300 recurring chromosomal translocations, of which more than 100 have been cloned (Kelly & Gilliland, 2002), providing important insights into the pathogenesis of the disease. Chromosomal aberrations are found in about half of all AML cases and are grouped into two major subtypes: balanced and unbalanced aberrations (Hiddemann et al., 2005). The biology of AML with balanced chromosomal translocations is fairly well understood, in contrast to the situation in AML with unbalanced aberrations (Hiddemann et al., 2005). Moreover, nearly half of all AML cases present a non-aberrant karyotype (Frohling et al., 2005; Hiddemann et al., 2005). In this category of AML, molecular analyses have revealed mutations in the *FLT3*, *CEBPA*, *KIT* and *NPM* genes, as well as *MLL* partial tandem duplication (Frohling et al., 2005; Hiddemann et al., 2005).

In AML, chromosomal translocations often result in loss-of-function mutations in transcription factors that are required for normal hematopoietic development (Kelly & Gilliland, 2002; Steffen et al., 2005; Tenen, 2003). The elucidation of the structure and function of leukemogenic genes, as well as the analysis of their occurrence in leukemia has led to a two-hit model of AML pathogenesis. According to this model, AML develops as a result of the concurrent emergence of two classes of genetic alterations (mutations or gene rearrangements). While class I mutations confer a proliferative and/or survival advantage to hematopoietic progenitors (Kelly & Gilliland, 2002; Steffen et al., 2005), class II mutations lead to impaired hematopoietic differentiation. The increase in cell proliferation and survival of leukemic blasts (class I mutations) is thought to be caused by alterations in receptor tyrosine kinase (RTK) signaling pathways. In this context, it has been demonstrated that mutations in FMS-like tyrosine kinase 3 (Flt3), c-Kit and Ras are frequent in AML (Kelly & Gilliland, 2002; Steffen et al., 2005). The activation status of growth

and survival pathways including phosphoinositide 3-kinase (PI3K)/Akt has also been found to be increased in AML blasts (Martelli et al., 2006). The impaired differentiation of AML blasts (class II mutation) is thought to occur through alterations in transcription factors required for normal myeloid cell differentiation (Kelly & Gilliland, 2002; Steffen et al., 2005; Tenen, 2003). In AML, these transcription factors are frequently the target of translocations, leading to the expression of fusion proteins such as AML1/ETO, CBFβ/SMMHC, PML-RARα, or MLL fusion proteins (Kelly & Gilliland, 2002; Steffen et al., 2005).

2.1.2. Genetic Alterations Involving Transcription Factors

Transcription factors are frequently targeted by balanced chromosomal translocations in AML. These include core binding factor (CBF), retinoic acid receptor alpha (RARα), and members of the HOX family of transcription factors (Kelly & Gilliland, 2002; Steffen et al., 2005; Tenen, 2003). Transcriptional coactivators, such as Creb-binding protein (CBP), p300, MOZ, TIF2, and MLL, are also targets of chromosomal translocations in AML (Kelly & Gilliland, 2002; Steffen et al., 2005). The targeted transcription factors are thought to be important for normal hematopoietic development. The consequence of the chromosomal translocations found in AML is loss-of-function and therefore impaired hematopoietic differentiation. For example, CBF is a heterodimeric transcription factor composed of a DNA-binding component, AML1, and the CBFß subunit which functions as a transcriptional activator of AML1. CBF controls the expression of genes that are critical for normal hematopoietic development. The three most common translocations involving CBF are the t[8;21], inv[16], and t[12;21] that result in expression of the AML1/ETO, CBFß/SMMHC, and TEL/AML1 fusion proteins, respectively. Each of these fusion proteins is a dominant negative inhibitor of CBF-mediated transcription. Transcriptional repression of CBF target genes by CBF-related fusion proteins is thought to be mediated through aberrant recruitment of the nuclear corepressor/histone deacetylase complex. Another example are fusion genes involving RARα, such as PML/RARα associated with the t[15;17] translocation in acute promyelocytic leukemia. Expression of the PML/RARα fusion protein results in a block of differentiation at the promyelocyte stage caused in part by the recruitment of the nuclear corepressor complex. This block is relieved by all-*trans*-retinoic acid (ATRA), which induces the release of the nuclear corepressor complex by binding to PML/RARα. In addition to these direct inhibitory effects on transcriptional activation, the fusion

proteins may also have additional roles in impairment of hematopoietic differentiation (Kelly & Gilliland, 2002).

Interestingly, in hematological clonal disorders in children with Down Syndrome (DS), mutations are commonly found in the gene encoding the transcription factor GATA-1 (Wechsler et al., 2002). The incidence of childhood acute leukemia is highly increased in children with DS, with a prevalence of acute megakaryoblastic leukemia (FAB M7) (Kurkjian et al., 2006). GATA-1 is known to be critical for normal development of erythroid and megakaryocyitc lineages, and the absence of GATA-1 promotes the accumulation of immature megakaryocytes (Magalhaes et al., 2006). The fact that mutations in GATA-1 occur in nearly every case of DS with a myeloproliferative disorder suggests that deregulation of GATA-1 is an essential step in the development of the disease in this particular patient group (Gurbuxani et al., 2004). Even though a direct link between trisomy 21 and the X-linked transcription factor GATA-1 have not been found so far, models including gene dosage effects have been proposed and trisomy 21 is thought to cooperate with the non- or dysfunctional GATA-1 in the multistep process in which progenitor cells acquire multiple genetic lesions (Gurbuxani et al., 2004).

2.2. Receptor Tyrosine Kinase Signaling

Receptor tyrosine kinases (RTKs) are central components of cell signaling networks and play a crucial role in normal physiological processes such as in embryogenesis and development. RTK networks control fundamental cellular activities including cell proliferation and survival, cell cycle control, metabolism, as well as cell shape and movement. They are able to detect, filter, and process a variety of environmental and intercellular factors. Numerous membrane spanning surface receptors have been identified and classified based on ligand preference, the induction of biological responses and their primary structure. Protein tyrosine kinases (PTKs) are endowed with intrinsic protein kinase activity that catalyzes the transfer of γ-phosphate of ATP to tyrosine residues of protein substrates (Hubbard et al., 1998). The receptor tyrosine kinases (RTKs) are one large family of PTKs that contain an intracellular catalytic protein tyrosine kinase domain, an extracellular ligand binding domain and regulatory sequences. More than 50 mammalian RTKs are known so far including the well known insulin receptor (IR), Fms-like tyrosine kinase 3 (FLT3), c-Kit, epidermal growth factor (EGF), platelet derived growth factor (PDGF), fibroblast

growth factor (FGF), colony-stimulating growth factor I (CSF-I) and insulin-like growth factor I (IGF-I) receptor (Hunter, 1998). A plethora of ligands such as peptides, proteins, lipids, or carbohydrates bind to and regulate the pleiotropic actions of RTKs. Tight control of the receptor activity is maintained by protein-tyrosine phosphatases, by other serine/threonine kinases, as well as autoregulatory mechanisms in the receptors. The extracellular binding of a specific ligand to a RTK induces conformational alterations that are translated across the membrane barrier resulting in the activation of its intracellular kinase activity (Schlessinger, 1988). In general, ligand binding induces allosteric interactions causing dimerization of receptor subunits through disulfide-bridges (Riedel et al., 1989) and autophosphorylation of specific tyrosine residues within their cytoplasmatic domains (Jiang & Hunter, 1999; Schlessinger, 1988). Dimerization-mediated receptor activation mechanisms have been described for receptors composed of heterotetrameric structures such as the IR, IGF-IR, PDGFR and the EGFR. Tyrosine autophosphorylation stimulates the intrinsic kinase activity of the receptor and leads to the generation of recruitment sites for downstream signal transducers. Src homology-2 (SH2) (Schaffhausen, 1995) or phosphotyrosine-binding (PTB) (Zhou & Fesik, 1995) domains are known phosphotyrosine-recognition domains present in various signaling proteins.

Activation of RTKs leads to rapid stimulation of phosphatidylinositol metabolism and the generation of multiple second messengers. One well characterized signaling pathway downstream of the IR and IGF-IR involves the insulin receptor substrates-1 to -4 (IRS-1 to -4) and the Src-homology collagen protein (Shc) isoforms as adapter molecules. The adapter proteins bind to the phosphotyrosine residues within the juxtamembrane region of the cytoplasmatic receptor and undergo subsequent tyrosine phosphorylation. Phosphorylation of the IRS adapter molecules on one hand triggers activation of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway (Myers et al., 1993; Vanhaesebroeck et al., 1997a; Yamamoto et al., 1992), whereas, on the other hand, the Shc adapter activates signaling by the Ras/Raf/MAPK pathway (reviewed in (Vanhaesebroeck et al., 1997a)). Generally, signals transduced through those signaling cascades have pleiotropic effects on cell behavior controlling cell proliferation, differentiation and cell migration, as well as apoptosis (Peruzzi et al., 1999). The PI3Ks phosphorylate phosphatidylinositol (PI) on the D-3 position of the inositol ring, producing distinct second messengers such as PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3 (PIP_3) (Katso et al., 2001). These lipid products activate signal transduction by diverse proteins including phosphoinositide-dependent kinase-1 (PDK1), forkhead (FKHR), glycogen synthase kinase-3 (GSK-3), tuberous sclerosis

complex 1 and 2 (TSC1, TSC2), BAD, Ras homologue enriched in the brain (Rheb), mammalian target of rapamycin (mTOR), 4E-binding protein (4E-BP) and ribosomal protein S6 kinase (S6K) (Katso et al., 2001). The Ras/Raf/extracellular signal-regulated kinase (Erk) cascade proceeds from Ras and its downstream effector Raf to Erk (p42/44 MAPK) (Seger & Krebs, 1995).

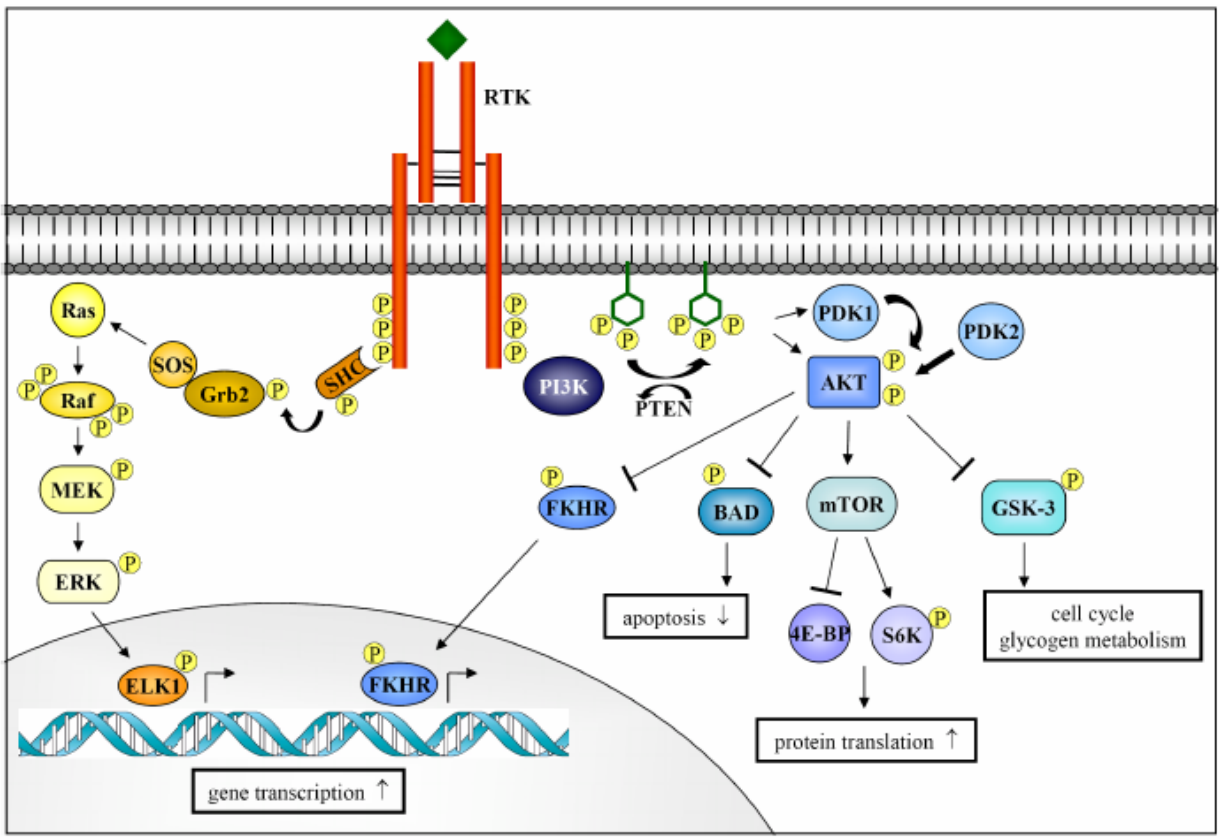

Fig. 1: Overwiew of RTK signaling. Signals are transduced via the mitogen-activated protein kinase (MAPK) or the phosphoinositide 3-kinase (PI3K) pathway. Key upstream regulators of MAPK signaling include growth factor receptor-bound protein 2 (Grb2), Son of Sevenless (SOS), Ras, Raf, MAPK/Erk kinase (MEK), extracellular signal-regulated kinase (ERK) and ELK1. Activation of the PI3K pathway is initiated by recruitment of the PI3K complex to the phosphorylated receptor. Downstream signals are transduced via phosphoinositide-dependent kinase 1 (PDK1), protein kinase B/Akt (AKT), the mammalian target of rapamycin (mTOR), 4E-binding protein (4E-BP) and ribosomal protein S6 kinase (S6K), forkhead (FKHR), BAD and glycogen synthase kinase-3 (GSK3).

Protein tyrosine phosphorylation was discovered more than 25 years ago (Hunter & Sefton, 1980) and protein tyrosine kinases, the signaling pathways they activate, as well as the mechanisms underlying their action and regulation have been widely studied. The importance of understanding the complexity of signaling networks, their regulation and the RTK interconnections has been highlighted by a large variety of molecular alterations and deregulations found in various human diseases including cancer.

2.2.1. Insulin-like Growth Factor I Receptor Signaling

The Insulin/IGF family of cell signaling factors comprises a phylogenetically conserved system with a critical role in growth and development of many tissues and the regulation of overall growth and metabolism. A high complexity is achieved by tight regulation of multiple proteins including three receptors (insulin receptor (IR), insulin-like growth factor I receptor (IGF-IR), and IGF-II/mannose 6-phosphate receptor (M-6-PR)), three ligands (insulin, IGF-I, and IGF-II), and six known types of circulating binding proteins (insulin-like growth factor binding proteins 1-6 (IGFBP1-6)) (De Meyts et al., 1994; Jones & Clemmons, 1995; Lee & Pilch, 1994). The IGF-IR is a transmembrane tyrosine kinase widely expressed in many human tissues and cell types with high homology to the IR. However, experimental data suggest distinct roles for these two receptors, namely control of cell growth, differentiation and apoptosis by the IGF-IR and the regulation of physiological processes by the IR (Blakesley et al., 1996; Patti & Kahn, 1998; Urso et al., 1999).

The IGF-IR is a heterotetrameric glycoprotein composed of two alpha and two beta subunits, post-transcriptionally linked by disulfide bonds (alpha2beta2). The alpha chain is extracellular, while the beta chain is composed of a short extracellular region, a single transmembrane domain, and a cytoplasmatic portion harboring the catalytic tyrosine kinase domain flanked by two regulatory regions and a juxtamembrane region that serves as a docking site for signaling molecules (Hubbard & Till, 2000). Activation of the receptor is achieved by binding of the specific ligand to the extracellular alpha subunit, triggering autophosphorylation of three tyrosine residues within the kinase domain of the beta subunit (Kato et al., 1993; Murakami & Rosen, 1991).

IGF-I and IGF-II are single-chain polypeptides with sequences 62% identical to pro-insulin. However, they are not proteolytically cleaved but remain linked in their mature form as four peptide domains (Daughaday & Rotwein, 1989; LeRoith et al., 1993). Unlike other peptide hormones, the production and storage of these growth factors is not organ- or cell type-restricted, even though the main fraction originates from the liver. Instead, IGFs may be produced by almost any human cell (Rosen, 1999). The half-lives, transportation and bioavailability of the IGFs circulating at high concentrations in the bloodstream and extracellular fluids are modulated by several high affinity IGFBPs (IGFBP1-6). More than 99% of the circulating IGFs is bound to IGFBPs or can be found in a ternary complex with IGFBPs and a third component, the acid-labile subunit (ALS) (Baxter, 1988). The IGFBP themselves are tightly regulated by tissue specificity,

cell or matrix association, phosphorylation and proteolysis by various proteases (Baxter, 2000; Ferry et al., 1999).

Both IGF-I and IGF-II interact with the IGF-IR, although IGF-I shows a much higher affinity than IGF-II. The closely related insulin, the main ligand for the IR, has an IGF-IR-binding affinity which is less than 1% of that of IGF-I (Denley et al., 2004). The intrinsic receptor for IGF-II, the M-6-PR, differs significantly from the IGF-IR, possesses no tyrosine kinase activity and was reported to target IGF-II for lysosomal degradation, without inducing a specific cell response (Kornfeld, 1992). The ability of the highly homologous IGF-IR and IR to form hybrid receptors by dimerization of their hemi-receptors further increases the complexity of the signaling system. Such IGF-IR/IR hybrid receptors have been reported to influence cell responses by altering the affinities of their growth factor ligands (Pandini et al., 2002).

The activated auto-phosphorylated IGF-IR undergoes major conformational changes, providing docking sites for the recruitment of substrate proteins in order to initiate intracellular signaling cascades. The so far best characterized signaling pathways involve the insulin receptor substrates-1 to -4 (IRS-1 to -4) and the Src-homology collagen protein (Shc) isoforms as adapter substrates. The adapter proteins bind to specific phosphotyrosine residues within the juxtamembrane region of the cytoplasmatic IGF-IR and undergo subsequent tyrosine phosphorylation. Phosphorylation of the IRS adapter molecules on one hand triggers activation of the PI3K/Akt signaling pathway (Myers et al., 1993; Vanhaesebroeck et al., 1997a; Yamamoto et al., 1992), whereas, on the other hand, the Shc adapter activates signaling by the Ras/Raf/MAP-kinase pathway (reviewed in (Vanhaesebroeck et al., 1997a)). Generally, signals controlled by the IGF-IR have pleiotropic effects on cell behavior controlling cell proliferation, differentiation and cell migration, but also regulating the apoptotic machinery (Peruzzi et al., 1999).

2.2.1.1. The Role of the IGF-IR in Human Cancer

In recent years, a growing body of evidence has arisen, suggesting a key role for IGF signaling in various types of human cancer. Since the signals emanating from activated IGF-IR regulate cell proliferation, survival, differentiation and transformation, mechanistic studies have been aimed at defining the role(s) of this receptor in the neoplastic phenotype. While the involvement of the IGF-IR in stimulating cell proliferation was an early finding, a number of studies performed in the last two decades have included experiments aimed at investigating the role of this receptor in the transformation of cells, as well as in metastatic events. Over-expression of the IGF-IR in NIH

3T3 cells lead to a fully transformed phenotype, including anchorage-independent growth and loss of contact inhibition, as well as rapid tumor formation in nude mice (Kaleko et al., 1990). More recently, a novel animal model was generated, involving transgenic expression of a fusion receptor that is constitutively activated by homodimerization (Carboni et al., 2005). The fusion gene was placed under the control of the mouse mammary tumor virus promoter, which drives transcription in the mammary and salivary glands of transgenic mice. The incidence and kinetics of tumor emergence in these animals suggest that the transgene is sufficient to initiate and maintain the transformation process. Transfection of a cell line established from a salivary gland adenocarcinoma with small interfering RNAs against the IGF-IR resulted in a 50% reduction in thymidine incorporation, emphasizing the effect of deregulated signaling via the IGF-IR on cell proliferation (Carboni et al., 2005). Alternatively, other studies have investigated the effect of decreased IGF-IR signaling. Mouse embryo fibroblasts (MEFs) homozygous for a targeted disruption of the *Igfr1* gene showed slower growth than wild-type cells in serum-containing medium and failed to form colonies in soft agar, even when stably transfected with activated Ras, a crucial downstream signaling mediator (Sell et al., 1994). Another approach to study impaired IGF-IR signaling was the generation of LCC6 cells, a metastatic variant of a breast cancer cell line, expressing a C-terminally truncated IGF-IR, which acts as a dominant-negative receptor (Sachdev et al., 2004). These cells showed decreased activation of downstream mediators, such as IRS-1 and IRS-2, as well as Akt. While proliferative responses of these cells to IGF-I or serum were not affected in vitro, they showed decreased anchorage-independent growth. Furthermore, analysis of xenograft tumor growth revealed that in contrast to wild-type cells, cells expressing the dominant-negative IGF-IR did not metastasize to the lungs, although they were equally aggressive locally when compared to wild-type cells (Sachdev et al., 2004).

While no cancer-specific mutations of the IGF-IR or its ligands have been described to date, a plethora of studies have provided evidence for a link between this signaling pathway and the risk of developing cancer (reviewed in (Khandwala et al., 2000)). The most common findings associated with deregulated IGF signaling are over expression of the IGF-IR or the establishment of autocrine or paracrine signaling loops (Table 1). While high expression levels of the IGF-IR have been found in breast and colorectal cancer (Nielsen et al., 2004; Peters et al., 2003), autocrine signaling loops are a more common phenomena, and have been reported in a wide variety of human malignancies (Guo et al., 1995; Nakanishi et al., 1988; Ohmura et al., 1990). Paracrine signaling has mainly been described for breast cancer, where stromal cells have been

shown to produce IGF-I and IGF-II (Gebauer et al., 1998; Yee et al., 1989). Population studies have further highlighted the importance of IGF signaling in some of the most common cancers. The most readily accessible components of the IGF signaling pathway are IGF-I, IGF-II and the IGFBPs, as their circulating concentrations can easily be determined from blood samples. It is important to mention that circulating IGF-I levels vary substantially between normal individuals (Greenspan & Gardner, 2001). Nevertheless, evidence from epidemiological studies has revealed a correlation between elevated IGF-I levels and an increased risk of cancer diagnosis (reviewed in (Pollak et al., 2004)). Although the population studies did not always come to the same conclusions, a recent systematic review of these results led to the interpretation that circulating IGF-I levels are indeed related to a risk of several common cancers (Renehan et al., 2004). The most significant correlation between increased levels of IGF-I and the risk of cancer diagnosis was found for prostate cancer and colorectal cancer (Chan et al., 2002; Giovannucci et al., 2000; Harman et al., 2000; Kaaks et al., 2000; Ma et al., 1999; Palmqvist et al., 2002; Stattin et al., 2000; Wolk et al., 1998). The majority of the studies observed that individuals with elevated levels of IGF-I had an increased risk of developing cancer. It is important to emphasize that the increases detected were modest, and therefore were not always confirmed in population studies, especially if cohort sizes were small (Lacey et al., 2001; Renehan et al., 1999). In the case of breast cancer, elevated levels of IGF-I have only been associated with an increased risk for premenopausal women, while no association between circulating levels and cancer risk was found for postmenopausal women (Hankinson et al., 1998; Keinan-Boker et al., 2003; Krajcik et al., 2002; Toniolo et al., 2000). Although elevated levels of IGF-I were detected in patients newly diagnosed with lung cancer (Yu et al., 1999), population studies were not able to confirm a direct link between plasma levels and cancer risk (London et al., 2002; Lukanova et al., 2001; Spitz et al., 2002). A possible explanation for this inconsistency is the strong influence of carcinogen exposure (e.g. cigarette smoking or asbestos) on the development of lung cancer.

Growth hormone (GH), produced in the pituitary, stimulates production of IGF-I. In patients with acromegaly, where GH levels are increased, retrospective studies suggest that GH hypersecretion modifies the progression of existing malignancies, particularly colon cancer (Orme et al., 1998; Webb et al., 2002). A study performed on GH-treated cancer survivors found no increased risk of disease recurrence or death in these patients. While the overall incidence of secondary malignancies was not increased, survivors of acute leukemia and lymphoma had an elevated number of secondary solid malignancies (Sklar et al., 2002). The relatively marginal effect of

GH/IGF excess on tumor onset and progression might indicate that the auto- and paracrine component of the mechanism is more important than the endocrine.

In summary, both mechanistic as well as epidemiological studies have provided valuable information concerning the role of IGF signaling in human cancers. Since this signaling pathway regulates essential processes such as cell proliferation, survival or differentiation, it might provide targets for the development of promising new therapeutic approach, which could possibly be combined with other classical treatment regimens.

2.2.2. Phosphoinositide 3-Kinases

The PI3Ks are a family of evolutionary conserved lipid kinases, playing a crucial role in controlling a wide variety of intracellular signaling events. PI3Ks phosphorylate phosphatidylinositol (PI) on the D-3 position of the inositol ring, producing distinct second messengers such as PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3 (PIP_3) (Katso et al., 2001). These lipid products are known to activate diverse target proteins involved in complex signaling cascades, ultimately resulting in the activation of cellular activities comprising cell growth, proliferation, survival and motility.

The family of PI3Ks identified in various species can be subdivided into three main classes (class I-III), based on structural similarity and *in vitro* substrate specificity (Katso et al., 2001) (overview given in Fig. 2). Class I_A includes p110α, p110β, and p110δ, known to form a heterodimeric complex with a p85, p55, or p50 (α or β) regulatory subunit. This adapter subunit contains two SH2 domains mediating their association with activated tyrosine kinase-coupled growth factor receptors (Katso et al., 2001). PIP_3 produced by class I_A PI3Ks activates the protein kinase phosphoinositide-dependent protein kinase-1 (PDK1), inducing the recruitment and activation of the key signal transducer protein kinase B (PKB)/Akt (Vanhaesebroeck & Alessi, 2000). Akt is involved in the regulation of the cell cycle and glucose metabolism through glycogen synthase kinase-3 (GSK-3) (Liang & Slingerland, 2003) and the modulation of cell growth and survival, as well as the control of the translational machinery through the mammalian target of rapamycin (mTOR), the ribosomal protein S6 kinase (S6K) and the 4E-binding protein (4E-BP) (Fingar & Blenis, 2004). Downstream events controlled by Akt further include the control of apoptosis through the regulation of proteins such as forkhead (FKHR), BAD, NF-κB and murine double minute gene 2 (MDM-2) (Downward, 2004) (overview given in Fig. 1).

Expression studies of the class I_A PI3K isoforms have pointed out an ubiquitous distribution of the subunits p110α and p110β in all human tissues, whereas p110δ is preferentially expressed in leukocytes (Chantry et al., 1997; Vanhaesebroeck et al., 1997a; Vanhaesebroeck et al., 1997b).

The class I_B PI3Ks is made up of one enzyme only, p110γ, which functions through heterodimer formation with a regulatory subunit p101 (Krugmann et al., 1999) or p84 (Suire et al., 2005). Activation of p110γ is controlled by receptors capable of activating heterotrimeric guanine nucleotide-binding proteins, termed G-protein coupled receptors (GPCRs) (Krugmann et al., 1999). The PI3K p110γ is thought to link GPCR signaling to PIP_3 production, governing cell motility in inflammatory cells such as macrophages and to some extent in neutrophils (Hirsch et al., 2000).

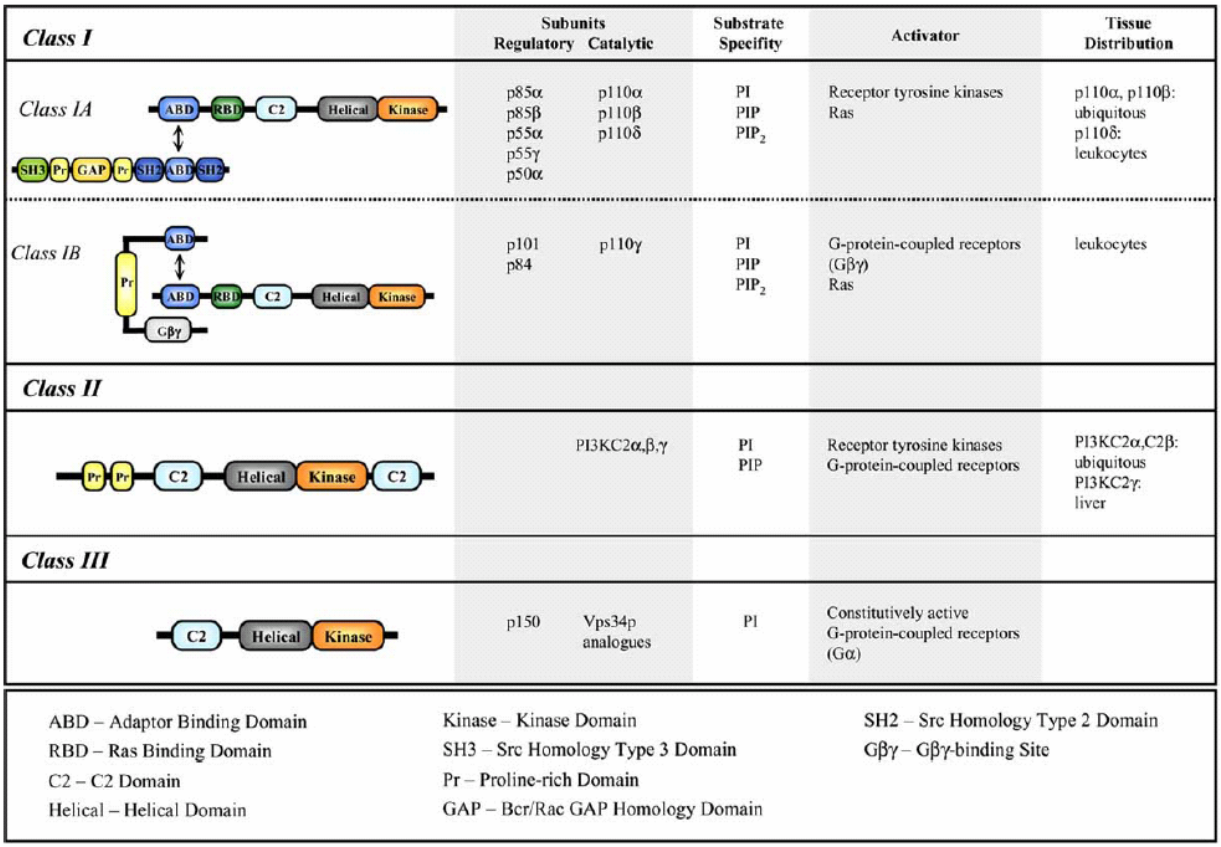

Fig. 2: Overview of PI3K isoforms.
(Figure from Doepfner et al.; *Recent Patents of Gene Sequences Relative to the Phosphatidylinositol 3-kinase / Akt Pathway and their Relevance to Drug Discovery* Recent Patents on DNA & Gene Sequences 2007, 1, 9-23 9)

The human class II of PI3Ks comprises the three isoforms PI3KC2α, PI3KC2β and PI3KC2γ (Katso et al., 2001). The hallmarks of class II family members are a substrate specificity restricted to PI and PI(4)P *in vitro*, and a C-terminal C2 domain. Although the precise cellular function of these enzymes remains generally unknown, recent reports have described class II PI3Ks as downstream transducers of activated polypeptide growth factor receptors (Arcaro et al., 2000; Brown et al., 1999). The class III PI3K includes a homolog of the yeast vesicular protein-sorting protein Vsp34 (Schu et al., 1993) and its major function is in intracellular trafficking events (Wurmser et al., 1999).

2.2.2.1. Inhibition of PI3K Signaling

As the PI3Ks have been identified as playing critical roles in distinct cellular signaling processes, a precise understanding of these kinases, their substrates, products and effectors is of particular interest. Anomalies in signaling cascades have been described in various human diseases and the knowledge of their components is of high importance in the search for therapeutic, diagnostic and screening applications. A number of attempts have been made to better understand the PI3Ks in general, as well as to gain insight into the relevance and specificity of the different isoforms. The first selective pharmacological PI3K inhibitors to have been described are wortmannin (Arcaro & Wymann, 1993; Powis et al., 1994), a compound that was originally isolated from soil bacteria and is toxic to fungi, and LY294002 (Vlahos et al., 1994), a morpholino derivative of quercetin, a naturally occurring bioflavinoid and broad spectrum kinase inhibitor. Both compounds have been shown to inhibit cell growth at concentrations that would be expected to inhibit class I PI3Ks. However, as these pharmacological inhibitors display little selectivity within the family of the PI3Ks and might moreover affect other kinases, further research has been aimed at developing compounds with improved specificity and pharmacokinetic properties.

2.2.2.2. Gene-Targeting Strategies

Gene-targeting strategies have been aimed at deleting specific PI3K isoforms and have uncovered key roles of the different enzymes in immunity, metabolism, cardiac function, cell proliferation and cancer susceptibility. Different knock-out mice have been generated with targeted deletions of genes encoding PI3K regulatory and catalytic subunits and have been phenotypically analysed (Vanhaesebroeck et al., 2005).

A knock-out mouse deleting the gene of the PI3K class I_A catalytic subunit p110α has been generated by targeting the p85-binding domain of the *PIK3CA* gene, leading to the loss of expression of this specific isoform (Bi et al., 1999). The *PIK3CA*$^{del/del}$ embryos showed a clear developmental delay and died between days 9.5 and 10.5 of embryonic development (Bi et al., 1999). In embryos missing the p110α enzyme, a profound proliferation defect could be observed, which has further been supported by a failure in replication of p110α-deficient fibroblasts in culture medium, even when supplemented with growth factors. As the developmental period between E9.5 and E10.5 is known for increased cellular proliferation, growth, and differentiation, it has been hypothesized that the intrauterine death of *PIK3CA*$^{del/del}$ embryos at this stage is caused by the incapacity to provide the increased demand in proliferation signals maintained by PI3K signaling through the p110α isoform (Bi et al., 1999). Moreover, mice deficient in the p110α subunit displayed multiple vascular defects (Lelievre et al., 2005). A role in the control of cell growth by p110α was further highlighted by an increase in heart size in transgenic mice expressing constitutively active p110α and a decrease in heart size by expression of a dominant-negative mutant form of this PI3K isoform in the heart (Shioi et al., 2000). The interpretation of knock-out data, however, has been complicated by the observation of an upregulation of other PI3K subunits after the deletion of one specific isoform. *PIK3CA*$^{del/del}$ homozygous embryos apparently increased the expression of the PI3K class I_A regulatory subunit p85/p55 raising the question of a contributing phenotypical effect by these adapter proteins. Further insight into the function of p110α has been given by the generation of mice carrying a knock-in mutation (D933A) that abrogates the p110α kinase activity (Foukas et al., 2006). Homozygocity for this mutation resulted in embryonic lethality while heterozygocity led to impaired signaling via the IRS proteins, key mediators of insulin, IGF-I and leptin action. As a result, the mutant mice displayed reduced somatic growth, hyperinsulinaemia, glucose intolerance, hyperphagia, and increased adiposity. Another study recently defined the p110α subunit as the critical lipid kinase required for insulin signaling in adipocytes and myotubes (Knight et al., 2006). The uncovering of p110α as a key intermediate in metabolic signaling raises concerns about potential mechanism-based side-effects and the therapeutic use of PI3K inhibitors .

The biological function of the PI3K class I_A catalytic subunit p110β has been studied by a partial deletion allele knock-out mouse (Bi et al., 2002). Targeting of this specific isoform resulted in very early embryonic lethality at the homozygous state. Zygotes with the *PIK3CB*$^{del/del}$ genotype

were nonviable very early after fertilization, leading to a deficiency of such embryos even at the blastocyst stage (Bi et al., 2002). Crossbreeding studies of p110β knock-out mice with p110α-deleted mice did not reveal a possible redundant function of these two class I_A catalytic subunits. However, a possible overlap in functions that might be manifested in subtle phenotypical abnormalities could not be ruled out so far (Bi et al., 2002).

PI3K class I_A catalytic subunit p110δ-deficient mice have been described by three different groups (Clayton et al., 2002; Jou et al., 2002; Okkenhaug et al., 2002). These studies revealed a major function of p110δ primarily in B and T lymphocytes and a failure of the knock-out mice to mount normal immune responses. The mice lacking p110δ showed a reduction in the amount of B1 and marginal zone B cells as well as lowered serum levels of immunoglobulins (Clayton et al., 2002). Furthermore, antigen receptor signaling in B and T cells was impaired and the mice were prone for the development of inflammatory bowel disease (Okkenhaug et al., 2002). However, despite the high level of p110δ expression in hematopoietic tissues, no significant differences have been found between wild-type and p110δ-deficient mice in the number or morphology of red blood cells and peripheral leukocytes, including neutrophils, eosinophils, basophils, monocytes, and lymphocytes, nor hemoglobin levels (Jou et al., 2002).

Knock-out mice of the PI3K class I_B catalytic subunit p110γ have been characterized by three different groups (Hirsch et al., 2000; Li et al., 2000; Sasaki et al., 2000b), describing phenotypes mainly affecting components of the innate immune response. Mice lacking p110γ showed an accumulation of defective neutrophils, exhibiting failure in their migratory capacity and reduced thymic cellularity. Moreover, chemoattractant-stimulated signal transduction was inhibited by impairment of PIP_3 production in p110γ-deficient cells (Sasaki et al., 2000b). In contrast to p110δ, the deletion of p110γ resulted in no effect on B cells. Instead, this isoform appeared to regulate proliferation and cytokine production of T lymphocytes (Sasaki et al., 2000b). In view of the impaired migration capability of p110γ knock-out macrophages towards a wide range of chemotactic-stimuli, a crucial role for macrophage accumulation in the inflammatory response was furthermore suggested (Hirsch et al., 2000). Phenotypical analysis of mice expressing a kinase-dead p110γ only partially reproduced the phenotype of knock-out animals (Patrucco et al., 2004). Whereas both mice exhibited an identical impairment in innate immune reactions, the p110γ-deficient mice additionally showed a basal enhancement of cardiac contractility and cardiac tissue damage upon pressure overload. It is therefore suggested that p110γ possesses a

kinase-independent activity controlling cardiac responses. A role of p110γ in the control of heart function has already been described by Crackower et al. (Crackower et al., 2002). These studies elucidated p110γ as a negative regulator of cardiac contractility through the inhibition of cAMP production.

Regarding the regulatory PI3K subunits, gene-targeting strategies have been aimed at disrupting the adapter subunits p85α, its splice variants p55α and p50α, as well as p85β. Ablation of the whole gene *PIK3R1*, which encodes p85α and the mentioned splice variants, resulted in perinatal lethality (Fruman et al., 2000). However, disruption of the *p85*α gene only lead to an impaired B cell development, a reduction in the number of mature B cells, reduced B cell proliferative responses and no T cell-independent antibody production (Fruman et al., 1999). Homologous deletion of the adapter subunit *p85β* gene resulted in a growth reduction of the knock-out mice compared to their wild-type littermates (Ueki et al., 2002). Surprisingly, muscle and adipocyte tissues of both, the p85α and p85β knock-out mice exhibited increased insulin-stimulated PI3K pathway activation and enhanced translocation of the glucose transporter isoform-4 to the plasma membrane, as well as hypoglycemia with decreased plasma insulin (Fruman et al., 2000; Ueki et al., 2002). Moreover, the mice showed enlarged muscle fibres, brown fat necrosis and calcification of cardiac tissue (Fruman et al., 2000).

2.2.2.3. PI3K and Diseases

As the PI3Ks are known to be involved in a wide spectrum of control mechanism within the cell, dysregulation of their function has been linked to various human diseases. Key roles of the PI3Ks have been described in the formation of tumors, metastasis, chronic inflammation, allergy and cardiovascular disease.

The importance of PI3K signaling in human cancer is highlighted by the fact that there are numerous oncogenes and tumor suppressor genes whose dysregulation advantages PI3K signaling and promotes malignant properties of cells (Vivanco & Sawyers, 2002). A prominent finding in this context are mutations in the tumor suppressor gene phosphatase and tensin homolog (PTEN) that have been described in various human tumors (Aggerholm et al., 2000; Ali et al., 1999; Katso et al., 2001; Vivanco & Sawyers, 2002). PTEN is a phosphatase that antagonizes the action of PI3K by dephosphorylating the D-3 position of polyphosphoinositides (Maehama & Dixon, 1998). The DNA sequence copy number of *PIK3CA*, the gene encoding the

p110α catalytic subunit of the PI3K located on chromosome 3q26, is frequently increased in ovarian cancers (Shayesteh et al., 1999). Moreover, recent reports have described activating mutations in the *PIK3CA* gene in a variety of other human cancers, including, breast, colon and medulloblastoma (Kang et al., 2005; Samuels et al., 2004). Although mutational alterations have predominantly been characterized for p110α so far, an increase in p110β activity has also been found in human colon cancer biopsies and adenocarcinoma cell lines (Benistant et al., 2000). Furthermore, a knock-down analysis of p110β in a prostate cancer mouse model suggested a requirement of this PI3K isoform for the formation of metastasis (Czauderna et al., 2003).

Gene-targeting strategies have furthermore uncovered a role of p110γ in colorectal adenocarcinoma (Sasaki et al., 2000a). Lack of p110γ significantly increased the incidence rate for spontaneous development of multifocal carcinomas and invasive adenocarcinoma in the colon of mice. In humans, protein expression analysis of p110γ revealed a loss of this protein in a high number of colon cancer cell lines as well as in primary adenocarcinomas isolated from the colon of human patients (Sasaki et al., 2000a).

As shown by different studies (Laffargue et al., 2002; Sasaki et al., 2000b), p110γ also plays a pivotal role in mediating leukocyte chemotaxis and activation, as well as mast cell degranulation, thus suggesting an involvement in inflammatory diseases. A specific contribution of the p110γ PI3K isoform to inflammation is supported by a recent study pointing out a protection of p110γ-deficient mice from rheumatoid arthritis (RA) (Camps et al., 2005). The mice were largely protected against collagen II-specific antibody-induced arthritis, correlating with the defective neutrophil chemotaxis in this knock-out model (Camps et al., 2005). Moreover, treatment of different RA mouse models with orally active small-molecule inhibitors of p110γ suppressed the progression of the joint inflammation and damage in these mice (Camps et al., 2005). Other studies, however, have described a positive role of the PI3Ks in the inflammatory response. In RA, the anti-inflammatory cytokine IL-10 is spontaneously produced by macrophages and infiltrating blood lymphocytes in the rheumatic joint (Brennan & Foey, 2002). An involvement of PI3K signaling in the regulation of IL-10 is known (Crawley et al., 1996) and further analysis have demonstrated that the inhibition of PI3K signaling suppressed the production of this cytokine (Foey et al., 2002).

The PI3K isoform p110δ has been shown to contribute to the inflammatory response as well (Clayton et al., 2002). Recent studies further supported this finding describing a contribution to

allergen-IgE-induced mast cell activation and vascular permeability which is a common characteristics of chronic inflammations such as asthma (Lee et al., 2006a). Pharmalogical inhibition of p110δ resulted in a significant reduction of serum levels of IgE, attenuation of airway inflammation and hyperresponsiveness, by preventing vascular leakage in murine asthma models (Lee et al., 2006a; Lee et al., 2006b).

Gene knock-out of the class I_A regulatory subunits in skeletal muscles of mice resulted in a significant reduction in muscle weight and fiber size. Moreover, these mice exhibited muscle insulin resistance and whole-body glucose intolerance (Luo et al., 2006). The p85 regulatory subunits are therefore thought to act as critical mediators of PI3K signaling in the regulation of muscle growth and metabolism and furthermore, to make an important contribution to symptoms of hyperlipidemia associated with human type 2 diabetes (Luo et al., 2006). Diabetes is associated with vascular complications, including the impairment of vascular function and alterations in the reactivity of blood vessels to vasoactive agents (Jarrett, 1989). It has been shown that PI3K signaling plays a role in vascular growth, proliferation and apoptosis and is implicated in modulating vascular smooth muscle cell contractility (Zdychova & Komers, 2005). A recent study demonstrated that selective inhibition of PI3K attenuated the development of diabetes-induced abnormal vascular reactivity in the carotid arteries of diabetic rats (Yousif et al., 2006).

The growing understanding of the biological functions of PI3Ks opened insights into the interconnection of signaling events and cellular actions and made these kinases interesting targets in clinical and research areas.

2.2.3. Alteration of RTK Signaling in AML

The increasing understanding of the pathogenesis of AML uncovered multiple genetic abnormalities necessary for the development of the disease. In the classical ‘two hit’ model, the second hit is thought to be caused by alterations in RTK signaling and various aberrantly regulated pathways have been described so far. Basically, there is an oncogenic potential in every receptor with tyrosine kinase activity. Structural modifications can lead to constitutive activation of RTKs, subversion of molecular control mechanisms and alterations in signal transduction. Deletions within the extracellular ligand binding domain alters ligand responsiveness, or eliminates negative control mechanisms that this structure might exert on the kinase domain. Even point mutations are able to induce overall ligand-independent conformational alterations

and hence activation of RTKs. Besides genetic alterations, over-expression of the wildtype receptor and/or autocrine receptor activation are known to play an important role in aberrant signal transduction.

FLT3 is the most frequently mutated gene in AML. About one third of all patients show either internal tandem duplications (ITDs) within the juxtamembrane domain (Nakao et al., 1996) of FLT3, or mutations within the activation loop, predominantly at position D835 (Yamamoto et al., 2001). FLT3 is a transmembrane receptor that has a crucial role in normal haematopoiesis. By signaling through the Ras/MAPK pathway the receptor exerts an important function in the control of cell proliferation. FLT3-ITD as well as activating loop mutations result in constitutive activation of the tyrosine kinase activity and of the whole signaling network (Gilliland & Griffin, 2002; Stirewalt & Radich, 2003).

Activating point mutations in the kinase domain of the human colony-stimulating factor (M-CSF or CSF-I) receptor FMS occur in 5-20% of patients with AML (Ridge et al., 1990). FMS is a cell surface RTK and specific point mutations in this receptor have been implicated in neoplastic transformation by inducing ligand independence and constitutive activation of the tyrosine kinase activity. The mutation at codon 969 of FMS is thought to alter a negative regulatory site of the receptor and to up-regulate the response to ligand binding, conferring a growth advantage to the cell (Ridge et al., 1990). Co-expression of M-CSF together with FMS has been described for a small subset of AML patients, implicating autocrine stimulation of the wildtype receptor (Rambaldi et al., 1988). Moreover, patients with myeloplastic syndrome harboring FMS mutations were shown to have a significantly increased frequency of transformation to AML (Padua et al., 1998).

Mutations in the RTK c-Kit have been preferentially associated with AML exhibiting either an inv(16) or a t(18;21) karyotype (Beghini et al., 2000). c-Kit encodes a transmembrane receptor that is activated SCF. Studies have shown that the levels of activation of c-Kit correlate with the rate of proliferation of myeloid leukemia cells and that receptor over-activation contributes to the excessive proliferation and aberrant differentiation of AML cells (Kuriu et al., 1991).

Mutations in the *RAS* gene family have been described in about one fourth of AML cases, predominantly in N-Ras, but also in K-Ras and, although very rarely, in H-Ras (Bartram et al., 1989; Farr et al., 1988). Primarily, mutations were found to affect the conversion of the active Ras-GTP form to the inactive Ras-GDP form leading to constitutively activated Ras and enhanced downstream signaling. Besides, over-activation of downstream transducers have been described as a result of FLT-ITD affecting the autonomous growth of AML cells (Hayakawa et al., 2000).

Moreover, increased activation of the Ras/MAPK pathway has been shown to be caused by a commonly occurring chromosomal translocation. BCR-ABL is a chimeric oncoprotein that binds to Grb-2, the substrate protein known to link receptor tyrosine kinases to Ras signaling. Coupling of BCR-ABL-Grb-2 to SOS subsequently induces activation of the signaling pathway (Pendergast et al., 1993).

FLT-ITD has furthermore been associated with increased activation of the Janus protein tyrosine kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. Constitutive tyrosine phosphorylation of the transcription factor STAT occurs in approximately 70% of AML cases, either through autophosphorylation of RTKs, or due to autocrine growth factor production (Birkenkamp et al., 2001). STAT signaling involves the control of diverse biological processes including cell proliferation, differentiation, development, and survival. Abnormal STAT signaling has been implicated in oncogenesis (Bromberg et al., 1999). Beside its role in FLT3 signaling, the JAK/STAT pathway has been associated with c-Kit-mediated ligand-independent survival and proliferation of AML cells. In fact, the c-Kit mutation Asp^{816} has been found to constitutively activate the JAK/STAT pathway (Ning et al., 2001). Moreover, chromosomal translocations resulting in the fusion of the *JAK2* and the *TEL* gene have been described in human leukemias. The TEL-JAK2 fusion protein confers constitutive tyrosine kinase activity and cytokine-independent proliferation to hematopoietic cells (Lacronique et al., 1997; Peeters et al., 1997).

An increasing body of evidence suggests a role of signaling by the IGF-IR through the PI3K/Akt/mTOR cascade in AML. IGF-I together with other cytokines have been described to be important for AML cell growth (Shimon & Shpilberg, 1995) and autocrine IGF-I production has been suggested to influence drug resistance in an AML cell line (Neri et al., 2003). Activation of the PI3K/Akt signaling pathway has furthermore been detected in blast cells from AML patients contributing to survival and proliferation of these cells (Grandage et al., 2005; Xu et al., 2003).

2.2.4. Targeting RTK Signaling in AML

Significant progress has been made over the past decades in the treatment of AML. Insights into the pathogenesis, detection of cytogenetic markers and classification of the disease have facilitated the definition of optimal therapy conditions. However, overall survival rates are still unsatisfactory and are highly influenced by complications resulting from therapy. Improvement of the current therapeutic approaches is thus urgently needed and major advances are hoped to arise from a more targeted therapy strategy. The increasing understanding of the high impact of receptor tyrosine

kinase signaling in AML and the discovery of specific alterations and dysregulation of signaling cascades sparked interest in specifically targeting RTK signaling in this disease. Besides, the growing number of molecularly targeted drugs displaying promising results in other human cancers further brought the focus onto targeting RTK signaling in AML and led to a plethora of preclinical and clinical trials.

Given the fact that a large number of AML cases show constitutive activation of one or more RTKs, the use of small molecule inhibitors targeting their tyrosine kinase activity appears to represent a great promise. The remarkable activity of imatinib mesylate (Gleevec) targeting the BCR-ABL fusion protein in chronic myeloid leukemia (CML) (Druker, 2003) further developed the interest in the field of moleculary-targeted therapies in leukemia. The major focus in AML has been directed towards FLT3 and c-Kit. The uncovering of other receptors important for AML cell biology and the development of a plethora of new small molecule inhibitors, however, has broadened the field of targeted research in this malignancy.

2.2.4.1. FLT3

FLT3 is a class III receptor tyrosine kinase expressed by immature hematopoietic cells. Its expression is important for the normal development of stem cells and the immune system (Gilliland & Griffin, 2002). The FLT3 ligand (FL) is a transmembrane protein that can be released as a soluble homodimeric protein. Both the membrane-bound, as well as the soluble form of FL are able to activate the tyrosine kinase activity of the receptor, and, synergistically with other hematopoietic growth factors, to induce proliferation of hematopoietic progenitor cells (Lyman & Williams, 1995). Expression of FLT3 is found in the majority of AML patients and a crucial role of this receptor in the survival and proliferation of leukemic blasts has been described in various studies (Carow et al., 1996). Molecular alterations in the FLT3 receptor were first described in 1996 by Nakao et al (Nakao et al., 1996). Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis revealed longer transcripts within the juxtamembrane domain of FLT3 in a large proportion of AML patients. This finding led to the discovery of commonly occurring ITDs within that region. A plethora of subsequent studies uncovered an overall frequency of FLT-ITDs of about one fourth of all cases of AML. More recently, in an additional cohort of AML patients, mutations within the activation loop of the kinase domain of FLT3 were found (Griffin, 2001; Yamamoto et al., 2001). Point mutations commonly occur at position 835 (Asp835) and have been reported in around 7% of AML cases so far. Taken together, the global incidence rate of altered FLT3 function

in AML patients caused either by FLT3-ITD, or by FLT3 activation loop mutation, represents almost one third of all cases. The high prevalence of activating mutations in this receptor therefore makes FLT3 one of the most interesting targets for molecularly based therapies.

Indoline tyrosine kinase inhibitors are known to inhibit the tyrosine kinase activity of a number of RTKs, such as FLT3, c-Kit, PDGFR, VEGFR or the FGFR. Pre-clinical studies have examined the effect on the FLT3 receptor and its signaling and initial clinical trials have been aimed at evaluating their biological activity in AML patients (Fiedler et al., 2003; Fiedler et al., 2005; Giles et al., 2003; Mesters et al., 2001; O'Farrell et al., 2003b; O'Farrell et al., 2004; Smolich et al., 2001; Yee et al., 2002). SU5416 and the related compound SU5614 were shown to inhibit the kinase activity of both the wildtype FLT3, as well as the mutated receptor. Furthermore, both compounds inhibited downstream signaling via the MAPK and the STAT pathway, markedly decreased cellular proliferation and increased apoptosis in AML (Smolich et al., 2001; Yee et al., 2002). In phase II clinical studies evaluating SU5416 in elderly AML patients, or patients with relapsed and/or refractory disease, single agent treatment had only minimal clinical activity. Partial remission and hematopoietic improvement was seen, however, in a small cohort of patients and these improvements were of short duration (Fiedler et al., 2003; Giles et al., 2003; O'Farrell et al., 2004). A significant decrease in blast cell count down to undetectable levels and stable remission after administration of SU5416 has only been described in a single case study of a patient with relapsed AML (Mesters et al., 2001). SU11248 is another oral multitargeted kinase inhibitor that targets FLT3 together with other RTKs. Pre-clinical studies showed *in vitro* activity against the wildtype, as well as the mutated FLT3 receptor and *in vivo* activity against xenograft FLT3-ITD AML tumors in engraftment mouse models (O'Farrell et al., 2003a). Phase I clinical trials underlined the action of SU11248 against the FLT3 kinase activity. Monotherapy of SU11248, however, only induced partial remission in a small number of AML patients for a short duration (Fiedler et al., 2005; O'Farrell et al., 2003b).

PKC412 is small molecule FLT3 inhibitor that was identified and characterized by Weisberg et al. (Weisberg et al., 2002). PKC412 selectively induces G1 cell cycle arrest and apoptosis in cell lines expressing mutant FLT3 by directly inhibiting its kinase activity. Progression of leukemia was inhibited in FLT3-ITD transduced mouse models making this inhibitor a valuable candidate for clinical trials in patients carrying mutant FLT3 receptors (Weisberg et al., 2002). Subsequent pre-clinical studies highlighted the potential use of this inhibitor against FLT3 kinase activity and reported an effect of PKC412 on the phosphorylation of the downstream targets Akt, Erk and

STAT5 (George et al., 2004). A phase II clinical trial in a small cohort of patients showed significant clinical benefit and a 50% decrease in peripheral blast counts in most patients with mutated FLT3, in line with inhibition of receptor autophosphorylation (Stone et al., 2005).

Levis et al. characterized CEP-701, an indolocarbazole derivative with potent activity against autophosphorylation of wild-type and constitutively activated FLT3 (Levis et al., 2002). CEP-701 inhibited the FLT3 downstream targets Erk and STAT5 and induced a cytotoxic effect in AML patient cells harboring FLT3-ITDs. Furthermore, CEP-701 prolonged survival in a mouse model of FLT3-ITD leukemia (Levis et al., 2002). A recent study substantiated the potential of CEP-701 in reducing cell viability and FLT3 phosphorylation in a large number of patient cells (Knapper et al., 2006b). *In vivo* hematological activity of CEP-701 has been studied in clinical phase I/II trials. Treatment of patients with refractory, relapsed, or poor prognosis AML expressing FLT3-activating mutations with CEP-701 revealed biological activity and a measurable clinical response including reduction in peripheral and bone marrow blood blasts in some patients (Smith et al., 2004). The clinical response was more pronounced in patients with mutated FLT3 than in those expressing the wild-type receptor (Knapper et al., 2006a).

Recently, tandutinib (MLN518) was identified as a novel, relatively specific FLT3 antagonist (Deangelo et al., 2006). Pre-clinical studies showed activity against FLT-ITD autophosphorylation and inhibition of AML cell proliferation *in vitro* (Kelly et al., 2002). A clinical phase II study reported evidence of antileukemic activity with a decrease in both peripheral and bone marrow blast count in a small cohort of patients treated with tandutinib (Deangelo et al., 2006).

2.2.4.2. c-Kit

c-Kit encodes another transmembrane protein with tyrosine kinase activity and is a proto-oncogene thought to play an important role in normal hematopoiesis. c-Kit expression has been detected in a large number of hematopoietic cell lines, in primary blasts of human AML patients, but to a much lower level in normal bone marrow cells (Wang et al., 1989). Tyrosine phosphorylation and activation of c-Kit is induced by binding of SCF and results in proliferation of human leukemia blast cells in a substantial fraction of AML cases. The constitutive, ligand-independent activation of c-Kit found in leukemic cells suggests that this receptor is involved in the excessive proliferation and aberrant differentiation of these cancer cells (Kanakura et al., 1993). Moreover, a direct correlation has been found between the levels of c-Kit tyrosine phosphorylation and the proliferation rate in AML cell lines (Kuriu et al., 1991). Mutations in c-Kit are thought to be

exclusively associated with core binding factor leukemias (CBF-AML). In addition to mutations within the tyrosine kinase domain of c-Kit at position 816, insertions and deletions have been described (Care et al., 2003; Gari et al., 1999).

Imatinib, also known as STI571 or Gleevec, has been shown to be a very promising small molecule inhibitor targeting the tyrosine kinase activity of the BCR-ABL fusion protein commonly found in CML. Moreover, the inhibitor was shown to effectively inhibit the PDGFR as well as c-Kit (Heinrich et al., 2000). Imatinib prevented autophosphorylation of c-Kit and activation of the downstream signal transducers MAPK and Akt. Furthermore, treatment of an AML cell line expressing the BCR-ABL fusion protein induced apoptosis and significantly increased the sensitivity to chemotherapeutical agents (Fang et al., 2000). In c-Kit-positive primary AML patient blasts, however, Imatinib exerted only marginal effects on cell growth (Scappini et al., 2001). Nevertheless, a pre-clinical study analyzing cells expressing c-Kit mutants cloned from AML patients reported a dose-dependent increase in apoptosis upon treatment with the inhibitor (Cammenga et al., 2005). In a first clinical phase II trial of patients with refractory or recurrent AML, Imatinib as single agent did not induce any significant beneficial responses (Cortes et al., 2003). A later phase II pilot study including a small cohort of c-Kit-positive patients, however, showed high efficacy of Imatinib resulting in the inhibition of c-Kit tyrosine phosphorylation and promising results in a subset of patients meeting the criteria for complete hematological remission (Kindler et al., 2004). Consistently, different case studies have described a potential benefit of Imatinib treatment for c-Kit-positive AML patients and carriers of specific c-Kit mutations (Cairoli et al., 2005; Ito et al., 2005; Jentsch-Ullrich et al., 2004; Kindler et al., 2003; Pompetti et al., 2006; Schittenhelm et al., 2003; Viniou et al., 2004; Yamaguchi & Konishi, 2003).

2.2.4.3. IGF-IR

The IGF-IR is a transmembrane tyrosine kinase widely expressed in many human tissues and cell types, with high homology to the IR. Activation of the receptor is achieved by binding of the IGFs to the extracellular domain, triggering autophosphorylation of three tyrosine residues within the kinase domain (Kato et al., 1993). In AML, signaling by the IGF-IR has not yet been extensively studied, however, expression of the IGF-IR was reported in human AML cells (Hizuka et al., 1987; Sukegawa et al., 1987). While mutations resulting in constitutive tyrosine kinase activity of the IGF-IR have not been described to date, over-expression of the receptor and/or the establishment of autocrine loops involving the ligands IGF-I and IGF-II have been reported in various human

cancers (Khandwala et al., 2000). The importance of IGF-I as a growth factor in combination with other cytokines has been described in human AML cells (Shimon & Shpilberg, 1995). A recent study further underlined the importance of IGF-IR signaling by showing an association between increased expression of IGF-I and resistance to the chemotherapeutical agent Cytarabine (Ara-C) in leukemia (Abe et al., 2006). Gene expression profiling of Ara-C-resistant human myeloid leukemia cells revealed an up-regulation of IGF-I. Subsequent analysis of AML cases uncovered higher IGF-I expression in patients with refractory disease after Ara-C therapy compared to patient cells analyzed at diagnosis (Abe et al., 2006). Consistently, a role of autocrine IGF-I production in drug resistance was previously suggested in an AML cell line (Neri et al., 2003). In addition, IGF-I signaling is known to play a crucial function in other hematological malignancies such as multiple myeloma (MM) (Yasui et al., 2006), and several anti-IGF-IR experimental therapies have been shown to inhibit multiple myeloma proliferation *in vitro* and *in vivo* (Mitsiades et al., 2004). Blocking the IGF-IR in the Ara-C resistant myeloid cell line described above inhibited cell growth and led to the induction of apoptosis, suggesting that the IGF-IR and its downstream signaling pathways may provide valuable novel targets to overcome Ara-C resistance in AML (Abe et al., 2006).

The increasing understanding of the importance of the IGF-IR signaling pathway in AML cell proliferation, viability and drug-resistance makes this system an interesting new molecular target for cancer therapy. Promising small molecule inhibitor have been generated and a wide variety of strategies are now available to target IGF signaling (Guerreiro et al., 2006).

2.2.5. Downstream Signal Transducers

Targeting pathways downstream of RTKs is another approach to down-regulate an overactivated signaling system with significant therapeutic advantages. The growing understanding of the complexity of RTK signaling networks has indeed revealed a number of promising target proteins contributing to altered signaling in cancer cells. As described above, deregulation of RTKs frequently induces over-activation of the PI3K/Akt, Ras/MAPK and JAK/STAT cascades in AML. Inhibitors targeting various signal transducer proteins including Akt and mTOR, Ras and MEK have been developed and are being tested in preclinical studies in various human cancers including AML.

2.2.5.1. PI3K/AKT

The PI3K pathway is essential for different physiological processes including transcription, translation, cell cycle progression and apoptosis. The PI3K cascade is the signaling system most frequently targeted by genetic alterations in human cancer, along with p53 and the retinoblastoma pathway. Genomic aberrations such as amplifications, rearrangements and mutations induce activation of the pathway, thus providing the tumor cells with a strong growth advantage (Osaki et al., 2004). The PI3Ks are a family of eight enzymes in humans, which are subdivided into 3 classes (I-III), based on sequence homology and substrate specificity (Vanhaesebroeck et al., 1997a). Class I_A and class II PI3Ks transduce signals from activated RTKs. These PI3Ks are also activated by oncogenic mutants of Ras, such as N- and K-Ras, which are found in AML. Several downstream targets of PI3K transduce their proliferative and anti-apoptotic signals, including Akt/PKB, PDK-1, FKHR, GSK-3β, TSC1 and TSC2, BAD, Rheb, mTOR, S6K, 4E-BP. The importance of the PI3K/Akt axis in AML cell progression has been highlighted by several studies (Kubota et al., 2004; Min et al., 2003; Xu et al., 2003; Zhao et al., 2004). Constitutive activation of PI3K was found in more than 50% of AML cases and the activation of Akt was significantly higher in spontaneously proliferating AML cells than in cells that did not proliferate spontaneously (Kubota et al., 2004). Moreover, constitutive phosphorylation of Akt at Ser473 negatively correlated with the overall survival rate of AML patients (Min et al., 2003).

The inhibition of PI3Ks by the generic inhibitor LY294002 resulted in reduced Akt kinase activity, dephosphorylation of Akt and BAD and an increase in apoptosis in AML cell lines (Xu et al., 2003; Zhao et al., 2004). PI3K inhibition in primary AML blasts resulted in significantly reduced clonogenic growth (Zhao et al., 2004). Moreover, inhibition of the pathway sensitized AML cells to chemotherapy-induced apoptosis (O'Gorman et al., 2000). A recent study examined the anti-leukemic effect of the novel small-molecule multiple kinase inhibitor KP372-1 in AML (Zeng et al., 2006). KP372-1 potently inhibited the kinase activity of Akt, PDK1and FLT3 in a dose-dependent manner. The inhibitor induced pronounced apoptosis in AML cell lines and primary blasts. Furthermore, KP372-1 decreased the colony-forming ability of primary AML blasts with minimal cytotoxic effects on normal progenitor cells (Zeng et al., 2006). The finding that normal hematopoietic progenitor cells were less affected by inhibitors targeting PI3K signaling than AML cells was previously described by others (Xu et al., 2003; Zhao et al., 2004) and further supports the feasibility of targeting this fundamental signal transduction network for AML therapy. The use of generic non-selective inhibitors such as LY294002 or wortmannin, however, has been shown to

cause severe side effects in xenograft models (Cheong et al., 2003) and the identification of more selective inhibitors is required. Recently, IC78114, a class I_A PI3K p110δ-selective inhibitor, was shown to efficiently suppress constitutive as well as FLT3-stimulated activation of Akt in AML blasts to the same extent as the non-selective generic PI3K inhibitor LY294002 (Sujobert et al., 2005). Moreover, the isoform-selective inhibitor suppressed AML cell proliferation without affecting the proliferation of normal progenitor cells.

2.2.5.2. mTOR

mTOR is a key regulator of growth and survival and is activated by various RTK receptors. Once activated, the serine/threonine kinase phosphorylates its downstream targets, the ribosomal S6K and 4E-BP. Aberrant activation of the mTOR signaling pathway in AML cells is supported by the finding of constitutive phosphorylation of S6K and 4E-BP in the majority of AML samples (Recher et al., 2005).

Rapamycin is a well known inhibitor of mTOR and is used clinically as an immunosuppressant and antiproliferative agent (Hidalgo & Rowinsky, 2000; Schreiber, 1991). Studies evaluating the effect of rapamycin in AML cells have reported marked activity of the inhibitor in down-regulating phosphorylation of S6K and 4E-BP (Hahn et al., 2005; Mohi et al., 2004; Recher et al., 2005) and inhibiting cell growth by blocking the cell cycle, particularly in very immature AML cell lines (Recher et al., 2005). Treatment of primary AML blasts with the mTOR inhibitor impaired their clonogenic properties, while normal hematopoietic progenitors were not affected. Moreover, rapamycin synergistically induced apoptosis in conjunction with protein kinase inhibitors (Hahn et al., 2005; Mohi et al., 2004). A pilot clinical study comprising a small number of patients with refractory, relapsed or poor prognosis AML showed significant responses suggesting that rapamycin could be of clinical interest for AML treatment. Partial remission or stabilization of the disease was reported in five out of nine patients analyzed (Recher et al., 2005). Evaluation of RAD001 (Everolimus), a rapamycin derivative, revealed only marginal effects on AML cell growth as a single-agent. Combined treatment together with the chemotherapeutical agent Ara-C, however, significantly enhanced the response of AML cells to the cytotoxic drug (Xu et al., 2003).

2.2.5.3. RAS/RAF/MEK/ERK

The MAPK signaling cascade is yet another important system that integrates extracellular stimuli and transduces them to cellular responses, such as proliferation, differentiation and survival. RTKs

signal through activation of the small GTP-binding protein Ras via the adapter molecule Grb2 and the guanine nucleotide exchange factor son of sevenless (SOS). Sequential stimulation of the cytoplasmatic proteins Raf, MEK and Erk, collectively known as MAPKs, finally results in the regulation of gene transcription by ELK1 and the STAT proteins (Seger & Krebs, 1995). The MAPK pathway was shown to be constitutively activated in a large number of AML cells, suggesting a pivotal role for this cascade in leukemogenesis (Kim et al., 1999; Towatari et al., 1997). N-Ras, but also K-Ras or H-Ras, are frequently mutated in AML cells causing dysregulation and activation of the signaling system (Bartram et al., 1989; Farr et al., 1988). As described above, altered signaling emanating from FLT3 receptors containing ITDs also relies, at least in part, on activation of MAPKs, thereby inducing autonomous growth of myeloid cell lines and primary AML blasts (Hayakawa et al., 2000). Moreover, the BCR-ABL chimeric oncoprotein that results from chromosomal translocation is yet another trigger for alterations in the MAPK pathway (Pendergast et al., 1993).

PD98059 and PD184352 are small molecule inhibitors that strongly reduce MAPK activity and profoundly impair growth and survival of AML cells (Milella et al., 2002). The primary effect of the inhibitors included cell cycle arrest followed by apoptosis in a significant percentage of leukemic blasts (Lunghi et al., 2003). The inhibitors abrogated the clonogenic properties of primary AML cells, but had only minimal effects on normal hematopoietic progenitors. Moreover, impairment of MAPK signaling sensitized leukemic cells to spontaneous, as well as drug-induced apoptosis (Milella et al., 2002; Wu et al., 2004). U0126, another potent MEK inhibitor, led to a highly significant induction of apoptosis in some AML cell lines and blasts, while no apparent responses were seen in others (James et al., 2003). Interestingly, a particularly pronounced effect on the most primitive types of leukemia, which are often found to be the most resistant to standard chemotherapy, was observed. A possible synergistic effect in combination with the chemotherapeutical agent Ara-C was also observed. Moreover, control cells were completely insensitive to the inhibitor (James et al., 2003). A phase I clinical trial addressed the question of efficacy of Sorafenib (BAY43-9006), a Raf-1 kinase inhibitor, in AML patients (Tong et al., 2006). Although no significant inhibition of the MAPK cascade, as assessed by phosphorylation of Erk, could be detected, c-Kit-mediated pathway activation was largely abolished upon treatment with the inhibitor. In fact, further analysis revealed an almost complete inhibition of c-Kit activity at high doses of the inhibitor, putting c-Kit forward as another target of Sorafenib (Tong et al., 2006).

Another approach to target the MAPK pathway is based on interfering with Ras protein function. Proper membrane localization of Ras proteins is a critical step for successful signal transduction. Binding of Ras to the plasma membrane, which is required for its full biological activity, is accomplished by diverse post-translational modifications of the protein, which are catalyzed by specific enzymes. Various inhibitors against these enzymes have been generated in order to impair the post-translational modification steps. The most significant progress has been made in the identification and characterization of farnesyltransferase inhibitors (FTIs). Treatment of tumor cells with these inhibitors results in various effects including alteration of cell cycle progression, induction of apoptosis, changes in cell morphology and inhibition of anchorage-independent growth (Sebti & Hamilton, 2000). Several FTIs have entered clinical trials for AML with promising results. Zarnestra (R115777) significantly inhibited the colony growth of human AML blasts and induced apoptosis. In combination with other drugs the effect of the FTI was even more pronounced (Korycka et al., 2004). Phase I and II clinical trials have described a promising biological activity of Zarnestra, inducing antileukemic responses in a subgroup of patients treated with the inhibitor (Karp et al., 2001; Lancet et al., 2006). Accordingly, evaluation of the FTI BMS-214662 showed strong evidence of antileukemic activity and a good tolerability of the compound in a cohort of patients with relapsed or refractory disease (Cortes et al., 2005).

3. RESULTS

3.1. Aims

Part I – Autocrine insulin-like growth factor-I signaling promotes growth and survival of human acute myeloid leukemia cells via the phosphoinositide 3-kinase/Akt pathway

RTKs and signaling cascades such as the PI3K/Akt pathway have been shown to play a crucial role in various human cancers. Promising new cancer therapies focus on pharmacological inhibitors selectively targeting signaling molecules of those survival pathways. Therefore, the role of RTK signaling and its biological function was investigated in AML. A panel of AML cell lines and patient blast cells were analyzed for the expression of various signaling proteins and their role in cellular responses. The main focus was laid on the IGF-I signaling network as high expression levels of the IGF-IR, the IR, as well as autocrine production of IGF-I were detected in the cells studied. Various apporaches were aimed at investigating the potential of targeting this signaling system including RNAi, neutralizing antibodies as well as the use of the novel IGF-IR kinase inhibitor NVP-AEW541 (Novartis) as an antitumor agent in AML. Besides, the involvement of downstream transducers of the IGF-IR on AML cell growth and survival was evaluated. Class I_A PI3K isoforms were specifically targeted in order to better understand their role in cellular responses and to investigate their potential as molecular targets for cancer treatment.
Together, we could describe a novel role for autocrine IGF-I signaling in the growth and survival of AML cells. The inhibition of the IGF-IR/PI3K signaling network in combination with chemotherapeutic agents may represent a novel approach to target human AML.

Part II – Targeting PI3KC2β impairs proliferation and survival in acute leukemia, brain tumours and neuroendocrine tumours

Eight mammalian PI3K isoforms exist which are subdivided into three classes. While much attention has been given to the class I isoforms, little is known about the role of class II PI3Ks in

human cancer. Therefore, the expression pattern and functions of the class II PI3K isoform PI3KC2β were investigated in tumor samples and cell lines from AML, glioblastoma multiforme (GBM), medulloblastoma (MB), neuroblastoma (NB), and small cell lung cancer (SCLC). Furthermore, the use of two novel isoform-specific pharmacological inhibitors (PI701, PI702) and RNAi to inhibit PI3KC2β was evaluated. The effect on signal transduction, cell growth and survival and the potential of PI3KC2β as a novel drug target in AML, brain tumors and neuroendocrine tumors was analyzed.

In summary, we could show that PI3KC2β contributes to proliferation and survival of AML, brain tumors and neuroendocrine tumors and plays an important role in the migratory capacity of highly motile breast cancer cells. Moreover, inhibition of this class II PI3K isoform sensitized AML and glioblastoma cells to chemotherapeutic agent.

Part III – Investigation of the molecular determinants of rapamycin sensitivity in acute myeloid leukemia cells

Protein expression analysis in a panel of AML cells revealed significant differences in the expression levels of mTOR. Therefore, the role of mTOR in AML cell growth and survival was investigated and cellular responses to the mTOR inhibitor rapamycin were compared in cells expressing high levels of mTOR ($mTOR^{high}$) and cells expressing low levels of mTOR ($mTOR^{low}$). Moreover, an RNAi screen was aimed at uncovering kinases which modulate the sensitivity to rapamycin. Screening a kinome siRNA library identified human kinases which strongly enhanced the anti-proliferative effect of rapamycin in AML cells and played a role in tumor cell survival. The use of specific inhibitors or shRNA targeting those kinases was further aimed at investigating their potential as molecular targets for AML treatment in combination with rapamycin.

Together, our data show an important role of mTOR for cancer cell proliferation and survival in a subclass of human AML cells. Preliminary results suggest that targeting certain RTKs (IGF-IR, FLT3, FGFR1) or signal transducers (SYK, ZAP70) in combination with rapamycin bears great potential for AML therapy.

Part IV – Novel role for insulin as an autocrine growth factor for malignant brain tumour cells

Atypical teratoid/rhabdoid tumors (AT/RTs) of the central nervous system (CNS) are childhood malignancies associated with poor prognosis. To gain better insight into the molecular background of this disease, the role of RTKs and their involvement in tumor gowth and survival was investigated in a panel of AT/RT and malignant rhabdoid tumor (MRT) cell lines. Over-expression of the IR and IGF-IR was detected in the cancer cells when compared to control normal brain tissue and insulin was shown to be secreted by AT/RT cells. Therefore, the potential of targeting the IR or IGF-IR signaling system in human AT/RT and MRT cell lines was investigated by the use of RNAi, neutralizing antibodies or NVP-AEW541. Several signaling proteins such as the receptors (IR and IGF-IR) as well as downstream transducers such as the class I PI3K isoform p110α were analyzed for their impact in tumor growth and survival and their potential as anti-proliferative target.
In short, a novel role for autocrine signaling by insulin and the IR in growth and survival of malignant human CNS tumor cells could be described.

Part V – Targeting the phosphoinositide 3-kinase isoform p110δ impairs growth and survival in neuroblastoma cells

Neuroblastoma (NB) is the most common extra cranial solid tumor occurring in children. Treatment outcome is still unsatisfying and a better understanding of the biology of this cancer is aimed at the identification of novel therapeutic targets in this cancer. A promising field of investigation is to target RTKs and their downstream mediators. Therefore, the role of RTK/PI3K signaling and the potential of targeting specific class I PI3K isoforms as a novel anti-proliferative approach was investigated in NB cells. Expression levels of PI3Ks was evaluated in primary human neuroblastoma samples and cell lines and the effects on cell survival and signal transduction was analyzed following isoform-specific down-regulation of p110α or p110δ in SH-SY5Y and LA-N-1 cells.
In conclusion, a novel function of the class I_A PI3K isoform p110δ in neuroblastoma growth and survival was uncovered.

3.2. Autocrine insulin-like growth factor-I signaling promotes growth and survival of human acute myeloid leukemia cells via the phosphoinositide 3-kinase/Akt pathway

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ORIGINAL ARTICLE

Autocrine insulin-like growth factor-I signaling promotes growth and survival of human acute myeloid leukemia cells via the phosphoinositide 3-kinase/Akt pathway

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Insulin-like growth factor (IGF) signaling plays an important role in various human cancers. Therefore, the role of insulin-like growth factor I (IGF-I) signaling in growth and survival of acute myeloid leukemia (AML) cells was investigated. Expression of the IGF-I receptor (IGF-IR) and its ligand IGF-I were detected in a panel of human AML blasts and cell lines. IGF-I and insulin promoted the growth of human AML blasts *in vitro* and activated the phosphoinositide 3-kinase (PI3K)/Akt and the extracellular signal-regulated kinase (Erk) pathways. IGF-I-stimulated growth of AML blasts was blocked by an inhibitor of the PI3K/Akt pathway. Moreover, downregulation of the class Ia PI3K isoforms p110β and p110δ by RNA interference impaired IGF-I-stimulated Akt activation, cell growth and survival in AML cells. Proliferation of a panel of AML cell lines and blasts isolated from patients with AML was inhibited by the IGF-IR kinase inhibitor NVP-AEW541 or by an IGF-IR neutralizing antibody. In addition to its antiproliferative effects, NVP-AEW541 sensitized primary AML blasts and cell lines to etoposide-induced apoptosis. Together, our data describe a novel role for autocrine IGF-I signaling in the growth and survival of primary AML cells. IGF-IR inhibitors in combination with chemotherapeutic agents may represent a novel approach to target human AML.

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Introduction

Acute myeloid leukemia (AML) accounts for approximately 70–80% of acute leukemia in adults. Treatment and outcome of the disease depend on several factors, including leukemia karyotype, molecular alteration and patient age. The 5-year survival rate ranges from 65 to 15%, drastically decreasing with the age at diagnosis. Chromosomal translocations frequently result in dysfunction of transcription factors needed for normal hematopoietic development. Additional mutations have been described in the receptor tyrosine kinases FLT3, c-Kit and c-Fms, as well as in N- and K-Ras.[1,2] The manifestation of AML is a combination of mutations conferring proliferative advantage, impaired differentiation and apoptosis.

Polypeptide growth factors have been shown to play a key role in AML proliferation and survival.[3–5] Human AML cells express a variety of growth factor and cytokine receptors that can be activated by mutation, overexpression and/or establishment of autocrine loops. Among these receptors are the polypeptide growth factor receptors FLT3, c-Kit, c-Fms, vascular endothelial growth factor receptor and fibroblast growth factor receptor.[3–8] Several potential anti-AML therapeutic approaches involving the FLT3 system have been reported.[9–11]

Insulin-like growth factor (IGF) signaling plays a major role in various human malignancies, including breast, colon and prostate cancer.[12] In leukemia, IGF signaling has not yet been extensively studied, although expression of the IGF-I receptor (IGF-IR) was reported in human AML cells.[13,14] Autocrine IGF-I production has been suggested to play a role in drug resistance in an AML cell line.[15] In addition, IGF-I signaling has a crucial function in other hematological malignancies such as multiple myeloma,[16] and several anti-IGF-IR experimental therapies were shown to inhibit multiple myeloma proliferation *in vitro* and *in vivo*.[17]

A critical intracellular signaling mediator of the IGF-IR is the phosphoinositide 3-kinase (PI3K)/Akt pathway.[18,19] Indeed, PI3K signaling is implicated in the control of cell proliferation, survival and motility/metastasis downstream of many different growth factor receptors.[20] The importance of PI3K signaling in human cancer is highlighted by the fact that mutations in the tumor suppressor gene *PTEN* occur frequently in human tumors.[20,21] PTEN is a phosphatase that antagonizes the action of PI3K by dephosphorylating the D-3 position of polyphosphoinositides.[22] Moreover, recent reports have described activating mutations in the *PIK3CA* gene encoding the catalytic p110α isoform of class I_A PI3K in a variety of human cancers, including, breast, colon and ovarian cancer.[23,24] Mutations in the PTEN gene have not been found in a high percentage of AML cases, although they were documented in AML cell lines.[21,25] Moreover, a recent screen comprising AML cases did not reveal any mutations in the gene encoding p110α,[26] suggesting a possible deregulation of other class I_A PI3Ks, namely p110β and p110δ in hematological malignancies. In support of this notion, constitutive activation of Akt/PKB has been reported by several studies in human AML blasts.[27,28] In the context of leukemia, altered PI3K signaling was also shown to play a role in the development of adult T-cell lymphoma.[29]

In the present report, we have investigated the expression pattern and biological functions of components of the IGF-IR signaling system in human AML blasts and cell lines. Moreover, we have evaluated the potential of the novel IGF-IR kinase inhibitor NVP-AEW541[30] as an antitumor agent in AML. Finally, we have investigated whether targeting downstream signaling mediators of the IGF-IR could suppress growth and induce apoptosis in AML cell lines. Our findings describe for the first time a role for autocrine signaling by IGF-I and the IGF-IR in growth, survival and chemoresistance of AML cells, which involves the PI3K/Akt pathway.

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Materials and methods

Reagents and antibodies

Antibodies and reagents were purchased from the following companies: IGF-IRβ, IRβ, p85α, p110β, p110δ, PTEN, Akt/PKB, Erk1/2, PARP, IGF-I and p-Tyr (Santa Cruz Biotechnology, Santa Cruz, CA, USA); p110α (clone U3A) (generous gift from Dr A Klippel); 16F-11 (Novus Biologicals, Littleton, CO, USA); actin, insulin (Sigma-Aldrich, St Louis, MO, USA); activated Akt/PKB (Ser473, Thr308), activated Erk1/2 (Thr202/Tyr204) (Cell Signalling Technology, Danvers, MA, USA); LY294002, IGF-I, IGF-IR neutralizing antibody (Calbiochem, La Jolla, CA, USA); IR neutralizing antibody (Biosource, Camarillo, CA, USA); si*GENOME* siRNA (Dharmacon, Lafayette, CO, USA); NVP-AEW541 (Novartis Pharma AG, Basel, Switzerland); TGX-221 (Prof. SP Jackson, Camarillo, CA, USA); IC87114 (ICOS Corporation, Indianapolis, IN, USA).

Cell culture

Human AML cell lines were grown in RPMI (Life Technologies Invitrogen, Carlsbad, CA, USA) with 10% (v/v) fetal calf serum (FCS) and penicillin/streptomycin/L-glutamine and passaged every 3–5 days by dilution. For growth factor stimulations, cells were incubated overnight in Optimem medium (Life Technologies/Invitrogen) and washed with serum-free medium before incubation with growth factors. Immortalized B cells[31] were cultured in RPMI with 20% (v/v) FCS and penicillin/streptomycin/L-glutamine. Heparinized peripheral blood or bone marrow samples were obtained from adult patients with AML. Each sample contained more than 90% blast cells. AML diagnosis was based on the criteria of the French–American–British Cooperative Group and immunophenotypic studies. Blast cells, isolated by centrifugation on Ficoll–Hypaque, were used immediately or kept frozen until use. Cell viability, as assessed by Trypan blue exclusion, was greater than 80% after 4–12 h of blast cell culture in RPMI medium/10% FCS. Immunophenotypic analysis was performed using a large panel of directly fluorescein isothiocyanate (FITC)- or phycoerythrin-conjugated mAbs reacting with leukocyte differentiation antigens CD-2, -3, -4, -7, -10, -13, -14, -15, -19, -20, -22, -33, -34, -41, -61, glycophorin A, HLA-DR, TdT or myeloperoxidase (antibodies were from Becton Dickinson, Franklin Lake, NJ, USA; Immunotech-Coulter, Miami, FL, USA; DAKO, Troy, MI, USA). Unconjugated mAbs were detected by indirect immunofluorescence using an FITC-conjugated goat anti-mouse antibody (Fab92 fragment; Tago). Double-immunofluorescence analysis was performed with an Epics flow cytometer (Coulter Electronics, Fullerton, CA, USA).

RT–PCR analysis

RNA was isolated using the RNeasy Mini Kit (Qiagen, Santa Cruz, CA, USA) from 1×10^6 cells. Reverse transcription–polymerase chain reaction (RT–PCR) was performed according to the QIAGEN OneStep RT–PCR protocol. Expression of IGF-1R, IR, IGF-I, IGF-II and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed using the following primers: IGF-IR-FP, 5′-ACTTCTGCGCCAACATCCTCA-3′; IGF-IR-RP, 5′-GGGAAATCAGGGGCAGTGAAGG-3′; IR-FP, 5′-GCTGAAGCT GCCCTCGAGGA-3′; IR-RP, 5′-CGGCCACCGTCACATTCCCA-3′; IGF-I-FP, 5′-GTGCTGCTTTTGTGATTTCTT-3′; IGF-I-RP, 5′-GTC TTGGGCATGTCGGTGTGG-3′; IGF-II-FP, 5′-ATGGGGAAGTC GATGCTGGTG-3′; IGF-II-RP, 5′-ACGGGGTATCTGGGGAAG TTG-3′; GAPDH-FP, 5′-AACGTGTCAGTGGTGGACCT-3′; GAP DH-RP, 5′-GGGTGTCGCTGTTGAAGTCA-3′.

ELISA assays

Enzyme-linked immunosorbent assay (ELISA) was performed on cell culture supernatant of AML blasts or cell lines kept in culture for 3 days. A fully serum-free culture system of RPMI containing bovine serum albumin (15 mg/ml), cholesterol (7.8 μg/ml) and transferrin (7.7 μM) was used for AML cell proliferation. Ninety-six-well plates (Costar EIA/RIA 96-well plates; Corning Incorporated, Corning, NY, USA) were coated with 100 μl of the supernatant, and antibodies specific for IGF-I or IGF-II were used to detect the presence of IGF-I and IGF-II, respectively. Horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Biosciences, Buckinghamshire, UK) was subsequently used for detection by addition of tetramethylbenzidine-H_2O_2 (TMB peroxidase EIA substrate kit; Bio-Rad Laboratories, Hercules, CA, USA), and the absorbance was measured according to the manufacturer's protocol.

Cell proliferation

AML cell lines (5×10^3 cells/well) were seeded in 96-well plates and grown for 72 h in serum (10%)-containing medium in the presence or absence of inhibitors. For growth factor stimulations, cells were incubated in Optimem medium (Life Technologies/Invitrogen). The number of viable cells was analyzed by means of an MTS assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), according to the manufacturer's instructions. Data are mean with s.d. from eight repetitions.

Apoptosis

For detection of apoptosis, AML cells were incubated for 16–24 h in the presence or absence of inhibitors and analyzed for caspase-3 and -7 activity using the Caspase-Glo 3/7 Assay (Promega) according to the manufacturer's instructions. Alternatively, the cells were lysed and caspase-3 activity was measured using the CaspACE Assay System (Promega). Additionally, samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blot with anti-poly(ADP-ribose) polymerase (PARP) antibodies.

Transient and stable expression in AML cells

U937 cells were transfected with small interfering RNA (siRNA) targeting the IGF-IR or IR and small hairpin RNA (shRNA) constructs[32] targeting or the parental pRetroSuper vector p110β (PIK3CB) or p110δ (PIK3CD) using the Amaxa Nucleofector system (Amaxa biosystems, Gaithersburg, MD, USA) according to the manufacturer's protocol. Cell Line Nucleofector Kit V was used and program V-001 applied. Constructs were from the library described previously.[32] After 48 and 72 h of transfection, cells were analyzed for cell proliferation by an MTS assay and lysed in cell lysis buffer in order to visualize protein expression by SDS–PAGE and western blotting. Alternatively, transfected cells were resuspended in fresh medium containing puromycin at 2 μg/ml and selected for 3–4 weeks. Puromycin-resistant U937 cells were maintained in media containing 2 μg/ml puromycin as stable transfectants.

SDS–PAGE and western blot analysis

Cellular lysates were prepared as described previously,[33] separated by SDS–PAGE, transferred to a hydrophobic polyvinylidene difluoride membrane (Hybond-P; Amersham Biosciences), and immunoblotted with various antibodies according to the manufacturer's protocol. Chemiluminescence was used

for visualization using the enhanced chemiluminescence western blotting detection reagents (Amersham Biosciences) according to the manufacturer's protocol.

Immunoprecipitation

Cells were kept in medium without serum and stimulated with IGF-I or insulin for 10 min at 37°C. The cells were then lysed in lysis buffer (1% Triton X-100) and incubated with Protein G-Sepharose beads (GE Healthcare, Piscataway, NJ, USA) and α-p-Tyr antibody for 3 h at 4°C with mixing. After washing with cold lysis buffer, samples were denatured in SDS–PAGE loading buffer and analyzed by western blot.

Colony-forming assay

Bone marrow $CD34^+$ cells (Lonza, Basel, Switzerland) were cultured at different densities (200, 500, 1000 per ml) in 1 ml human methylcellulose complete media (HSC004; R&D Systems, Minneapolis, MN, USA) with increasing concentrations of NVP-AEW541. Each condition was evaluated twice in duplicates. Colonies, defined as aggregates >50 cells, were scored after 14 days incubation at 37°C in a fully humidified atmosphere with 5% CO_2.

Results

Characterization of the expression of the IGF-IR, IGFs and signaling intermediates in a panel of human AML blasts and cell lines

In view of the crucial role of IGF-I signaling in a variety of human cancers, we have studied the expression and biological functions of components of the IGF-IR pathway in human AML cells. A panel of 20 primary human AML blasts (Supplementary Table 1) and 7 low passage AML cell lines was analyzed for the expression of the IGF-IR and insulin receptor (IR) by western blot and RT–PCR analysis of cell lysates. The IR was studied in parallel with the IGF-IR, since the response of cancer cells to anti-IGF-IR inhibitors may also be modulated by expression of the related IR. Nonleukemic human bone marrow cells were used as a normal control for the western blot analysis. Moderate to high IGF-IRβ expression levels were found in 16/20 of the blasts and in all the cell lines (Figure 1a and Supplementary Figure 1a). The IR was expressed in 19/20 blasts with overexpression in 17 of the 19 cases, as compared to nonleukemic bone marrow cells, and in cell lines such as U937 and THP1. Expression analysis of class I_A PI3K isoforms revealed a consistent overexpression of the regulatory subunit p85α in AML blasts and cell lines, while expression of the catalytic p110α subunit was restricted to a small number of blasts and the KG-1 cell line (Supplementary Figures 1a and b). Consistently, RT–PCR analysis revealed only low mRNA levels of this isoform in AML cell lines (data not shown). In contrast, the class I_A PI3K catalytic isoforms p110β and p110δ displayed a broader expression pattern in AML blasts and cell lines, and overexpression was found in more than 50% of the blasts. The western blot analysis revealed no significant differences in expression of the downstream signaling intermediate extracellular signal-regulated kinase (Erk) (Supplementary Figure 1c), while Akt/PKB levels were elevated in the AML blasts, when compared to nonleukemic bone marrow cells. Constitutive activation of Akt/PKB was detected in all AML blasts and cell lines, while some of the blasts and cell lines also displayed detectable activation of Erk1/2.

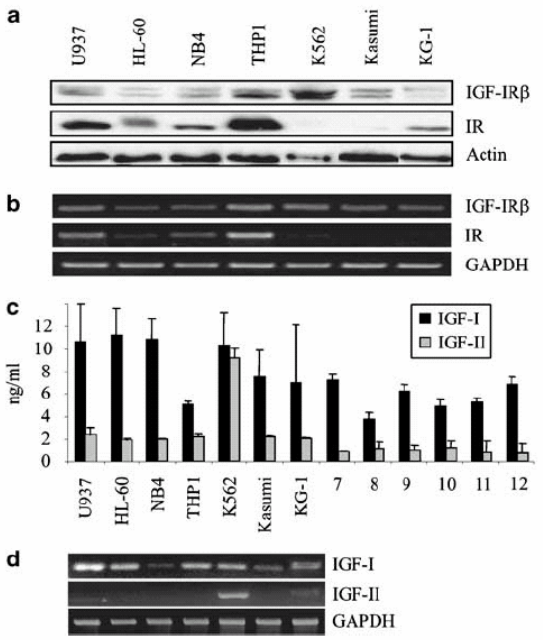

Figure 1 Expression of the insulin-like growth factor I receptor (IGF-IR), insulin receptor (IR) and insulin-like growth factors (IGFs) in human AML patient blasts and cell lines. (**a**) Equal amounts of lysates from seven AML cell lines (U937, HL-60, NB4, THP1, K562, Kasumi, KG-1) were analyzed by western blotting with antibodies specific for the indicated proteins. Actin was used as a loading control. (**b**) Analysis of IGF-IR and IR mRNA levels by RT–PCR in leukemic cell lines. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed as a control. (**c**) Measurement of IGF-I and IGF-II secretion in the medium by leukemic cell lines and patient blasts (7–12; FAB M1, M2, M4, M5) by ELISA. Supernatant of cells kept in serum-free medium for 72 h was analyzed using antibodies specific for IGF-I and IGF-II. The background absorbance of the cell culture medium was subtracted from each sample. (**d**) Analysis of IGF-I and IGF-II mRNA levels in leukemic cell lines by RT–PCR.

The expression of the ligands of the IGF-IR was then investigated in the AML blasts and cell lines by RT–PCR and ELISA. AML cell lines grown under serum-free conditions secreted detectable levels of IGF-I into the culture medium, as assessed by ELISA (Figure 1c), which correlated with RT–PCR analysis revealing broad expression of the IGF-I mRNA in most of the AML cell lines (Figure 1d). In contrast, IGF-II expression was restricted to the K562 cell line. The levels of IGF-I and IGF-II secreted in the supernatant of AML cell lines were in the range of 6–12 and 10 ng/ml, respectively. At these concentrations, IGF-I was able to activate downstream signaling events (Figure 2c), implying that the amounts of IGF-I secreted by AML cells are biologically relevant. IGF-I secretion was also detected in primary AML blasts, confirming the results obtained in cell lines (Figure 1c). Thus, primary AML blasts and cell lines express the IGF-IR, IR, the ligand IGF-I and downstream signaling mediators such as class I_A PI3K isoforms.

IGF-I and insulin promote growth of AML blasts through the PI3K pathway

The ability of IGF-I and insulin to promote growth of human AML blasts *in vitro* was then investigated. Treatment of isolated

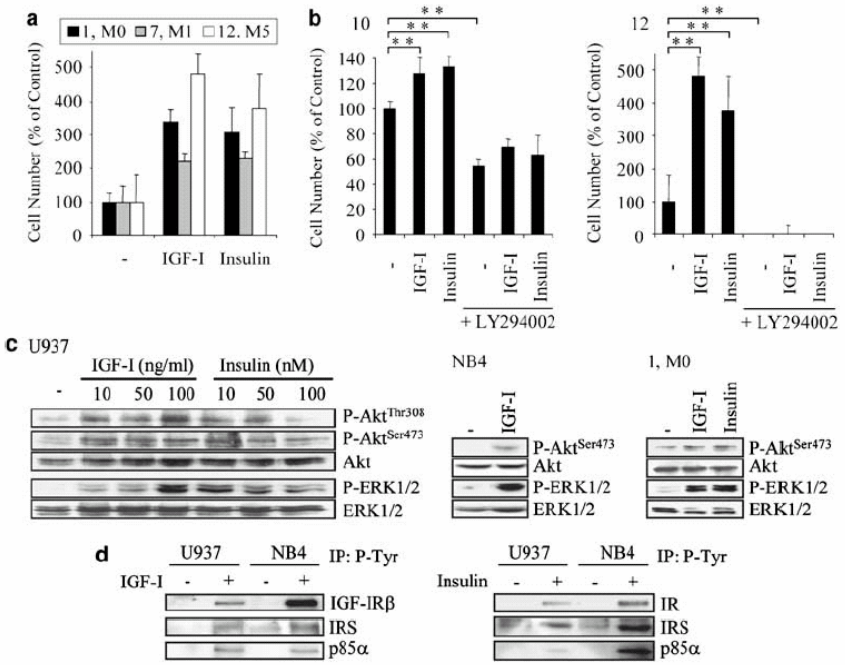

Figure 2 Insulin-like growth factor I (IGF-I) and insulin stimulate proliferation of AML cells through activation of the PI3K/Akt pathway. (**a**) Cell proliferation of AML patient blasts by stimulation with IGF-I (25 ng/ml) or insulin (50 ng/ml). (**b**) Inhibition of IGF-I- or insulin-stimulated AML cell proliferation by the PI3K inhibitor LY294002 (10 μM) in AML patient blasts. *$P<0.05$ or **$P<0.01$ by analysis of variance test. (**c**) Serum-starved U937, NB4 or primary blasts were stimulated with different concentrations of IGF-I or insulin for 10 min and evaluated by western blotting for the phosphorylation status of Akt/PKB (Thr308; Ser473) and Erk1/2 (Thr202/Tyr204). (**d**) Anti-phosphotyrosine immunoprecipitation (P-Tyr) from IGF-I- or insulin-stimulated U937 and NB4 cells. Cell lysates were analyzed by western blotting for IGF-IR, insulin receptor (IR), insulin receptor substrate (IRS) and p85α.

blasts with IGF-I or insulin resulted in a two- to fourfold increase in AML blast growth after 48 h in serum-containing medium (Figure 2a). The optimal concentrations of IGF-I and insulin for promotion of blast growth were 25 and 50 ng/ml, respectively. These results were observed in a panel of human AML blasts including AML M0, M1 and M5. To investigate whether the PI3K pathway was involved in the growth-promoting effects of IGF-I and insulin, AML blasts were treated with the pharmacological inhibitor LY294002 in combination with growth factors. The pharmacological inhibitor impaired IGF-I- and insulin-stimulated growth of primary AML blasts *in vitro* (Figure 2b).

The activation of the PI3K and Erk pathways by IGF-I and insulin was then investigated in AML cells by monitoring the phosphorylation status of the downstream targets Akt and Erk1/2. Optimal concentrations of IGF-I (100 ng/ml) and insulin (10–50 nM) resulted in the rapid induction of Akt phosphorylation on Thr308 and Ser473, as well as in phosphorylation of Erk1/2 in U937 cells (Figure 2c). Comparable results were also obtained in the NB4 cell line and in AML blasts. Thus, IGF-I and insulin promote AML cell growth by activating the PI3K/Akt signaling pathway. Stimulation of U937 and NB4 cells with IGF-I or insulin induced the recruitment of the receptors to phosphotyrosine-containing signaling complexes also containing the insulin receptor substrate-1 and the PI3K regulatory subunit p85α (Figure 2d).

Downregulation of the PI3K isoforms p110β and p110δ impairs AML cell growth, induces apoptosis and impairs Akt activation by IGF-I

The results from the expression analysis of PI3K isoforms in AML blasts and cell lines had revealed consistent expression of the PI3K isoforms p110β and p110δ (Supplementary Figure 1b), indicating a possible role for these enzymes in AML cell responses. Moreover, inhibition of PI3K activity with LY294002 impaired growth of AML blasts induced by IGF-I and insulin (Figure 2b). An RNA interference (RNAi) approach was used to specifically downregulate the expression of p110β and p110δ in U937 cells. Western blot analysis confirmed the specific downregulation of the PI3K isoforms upon transfection with the corresponding (short hairpin) shRNA construct (Figure 3a). U937 cells stably transfected with either p110β and p110δ shRNA displayed reduced growth in serum-containing medium (Figure 3b). Moreover, the p110β and p110δ shRNA-transfected cells were more sensitive to apoptosis induced by serum withdrawal, as assessed by the induction of caspase-3 activation (Figure 3b).

Resistance of human AML cells to chemotherapy is a frequent cause of treatment failure. Therefore, we next investigated whether downregulation of p110β or p110δ could sensitize human AML cells to the action of chemotherapeutic agents. Treatment with cytarabine (Ara-C) or etoposide significantly enhanced the suppression of cell growth in p110δ shRNA-

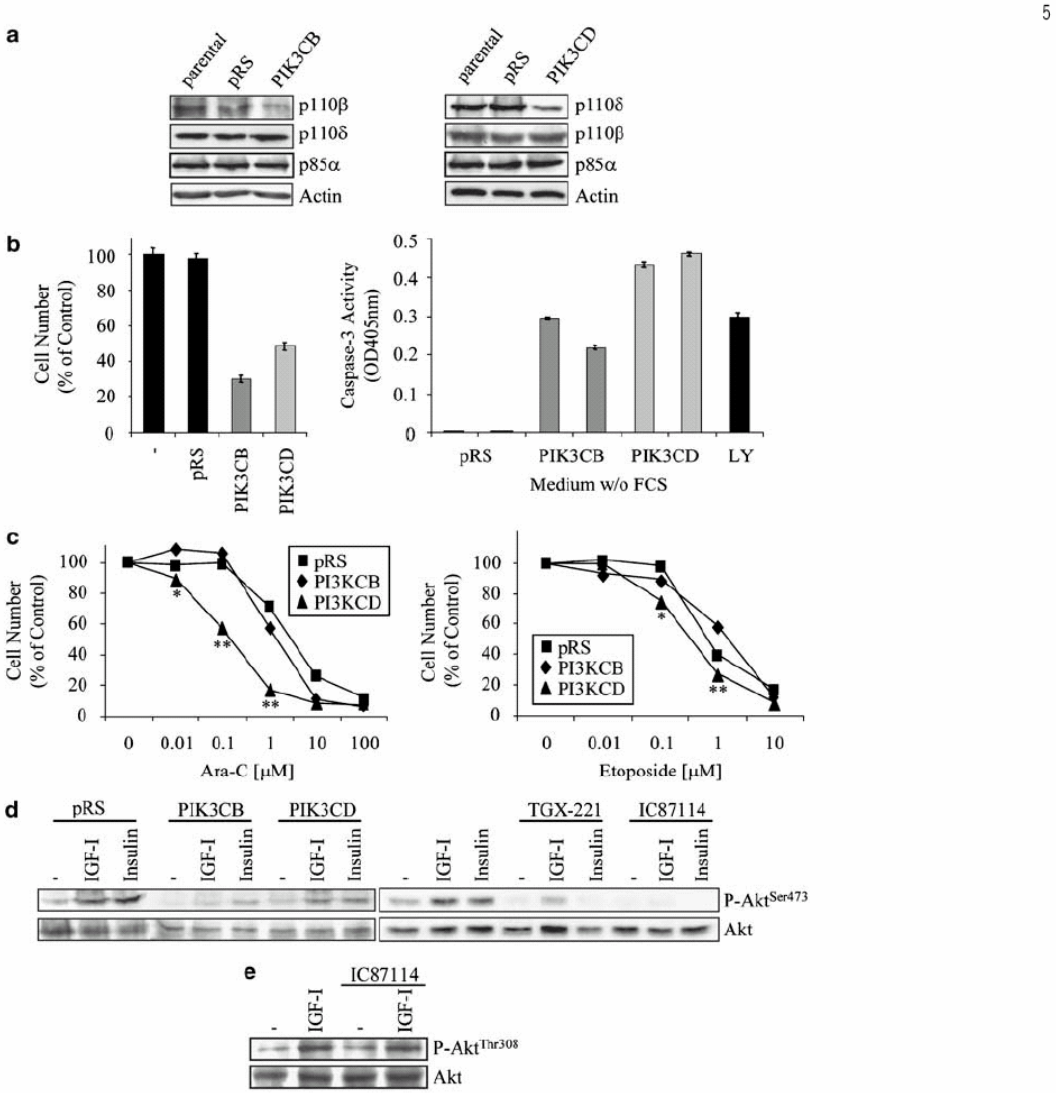

Figure 3 Downregulation of PI3K isoforms p110β or p110δ reduces cell viability of AML cells and sensitizes the cells to chemotherapeutical agents. (**a**) U937 cells were stably transfected with shRNA against the class I_A PI3K isoforms p110β (PIK3CB) or p110δ (PIK3CD) or the empty vector (pRS) as a control. Downregulation of the protein was visualized by western blot analysis using isoform-specific antibodies. (**b**) U937 cells transfected with shRNA against p110β (PIK3CB), p110δ (PIK3CD) or the empty pRetroSuper vector were assayed for cell proliferation by MTS assay (left). Apoptosis was analyzed in serum-free medium (RPMI W/O FCS or OPTIMEM) by caspase-3 activity measurement. LY294002 (10 μM) was used as a control (right). (**c**) Cell proliferation rate was analyzed in U937 cells transfected with shRNA against p110β (PIK3CB), p110δ (PIK3CD) or the empty pRetroSuper vector incubated with increasing concentrations of cytarabine (Ara-C) or etoposide. *$P<0.05$ or **$P<0.01$ by analysis variance test. (**d**) U937 cells transfected with shRNA against p110β (PIK3CB), p110δ (PIK3CD) or the empty pRetroSuper vector (left) or pretreated with PI3K isoform-specific inhibitors against p110β (TGX-221; 10 μM) or p110δ (IC87114; 10 μM) (right) were kept in medium without serum, stimulated with insulin-like growth factor I (IGF-I) or insulin and analyzed by western blotting for the phosphorylation status of Akt/PKB (Ser473). (**e**) IC87114-treated KG-1 cells were stimulated with IGF-I and analyzed by western blotting for the phosphorylation of Akt/PKB (Thr308).

transfected cells at concentrations in the range of 0.01–1.0 μM (Figure 3c).

To investigate whether p110β or p110δ transduce signals from the activated IGF-IR or IR in AML cells, activation of Akt was analyzed in shRNA-transfected cells. U937 cells transfected with shRNA targeting p110β and p110δ displayed impaired activation of Akt in response to IGF-I or insulin (Figure 3d). To confirm these findings, a pharmacological approach using isoform-specific p110β or p110δ inhibitors was also used in U937 cells. The p110β-specific inhibitor TGX-221[34] and the p110δ-specific inhibitor IC87114[35] effectively impaired activation of Akt by IGF-I or insulin (Figure 3d). IC87114 did not affect IGF-I-stimulated Akt phosphorylation in KG-1 cells expressing very low levels of p110δ (Figure 3e), confirming the selectivity of the inhibitor.

Together these data show that the class I_A PI3K isoforms p110β and p110δ play a major role in AML cell growth and survival. In addition, p110δ appears to play a selective role in chemoresistance in AML cells. Moreover, p110β and p110δ transduce signals from the activated IGF-IR leading to Akt activation in AML cells.

Pharmacological inhibitors of the IGF-IR or neutralizing antibodies impair AML cell growth and induce apoptosis

The ability of the specific IGF-IR kinase inhibitor NVP-AEW541[30] to block AML cell growth in serum-containing medium was then investigated. NVP-AEW541 potently suppressed growth of NB4 and Kasumi cells ($IC_{50} = 0.7$ and $0.4\,\mu M$) after 72 h, but was less efficient at inhibiting growth of HL-60 ($IC_{50} = 6.3\,\mu M$), U937 ($IC_{50} = 7.6\,\mu M$) and THP1 ($IC_{50} = 9.7\,\mu M$) (Figures 4a and b). The inhibitor was also able to suppress growth of AML blasts isolated from patients (Figure 4a) with IC_{50} values in the range of 2.2–13 μM. Nonleukemic bone marrow cells or immortalized B cells were comparatively resistant to NVP-AEW541. The expression levels of the IGF-IRβ, which is the target of NVP-AEW541, did not correlate with the sensitivities of the AML cell lines to the inhibitor (Figures 1a and 4a). The highest levels of IR expression were found in some of the cell lines with reduced sensitivity to the inhibitor such as U937 and THP1. In order to rule out potential cytotoxic effects of NVP-AEW541 on normal hematopoiesis, the inhibitor was tested at different concentrations on $CD34^+$ hematopoietic progenitors from a healthy donor. Whereas high concentrations

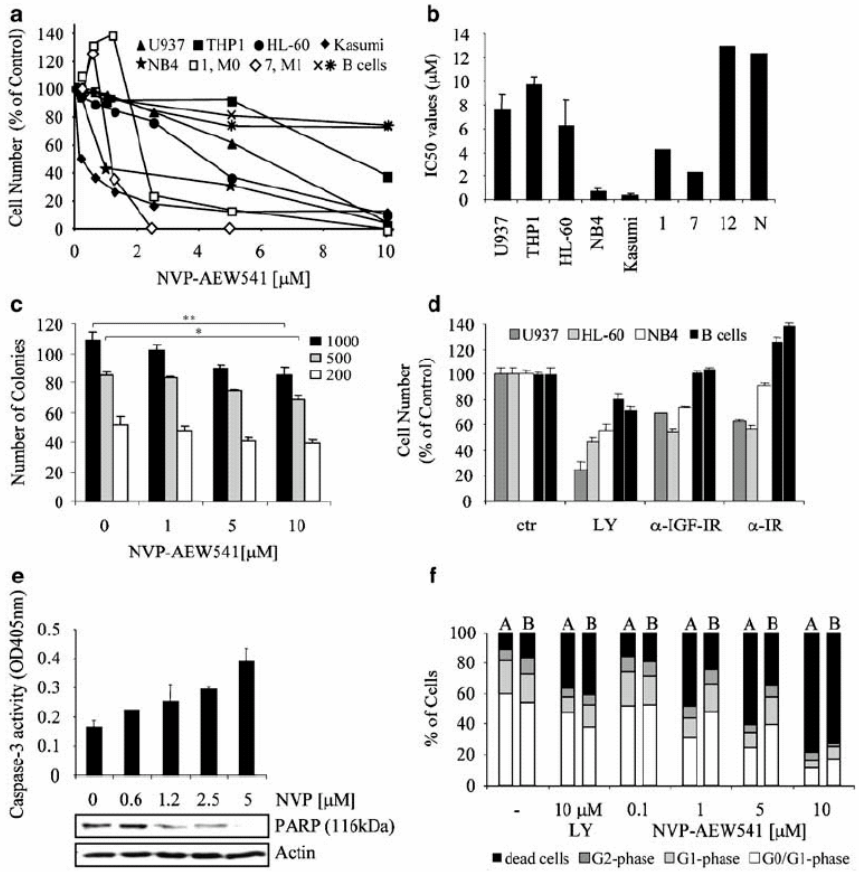

Figure 4 Inhibition of AML cell proliferation and induction of apoptosis by an insulin-like growth factor I receptor (IGF-IR) kinase inhibitor or an anti-IGF-IR neutralizing antibody. (**a**) Cell proliferation rate analyzed by MTS assay in AML cell lines, patient blasts and immortalized B cells incubated with increasing concentration of NVP-AEW541. (**b**) Sensitivity of AML cell lines, patient blasts and Ficoll-purified normal bone marrow cells (*N*) to the IGF-IR kinase inhibitor NVP-AEW541 by evaluation of the IC_{50} values from proliferation assays. (**c**) Colony growth of NVP-AEW541 treated $CD34^+$ hematopoietic progenitor cells in semisolid growth medium. Cells were plated at different densities (200, 500, 1000 cells in 1 ml medium), treated with increasing concentrations of the inhibitor (1, 5, 10 μM) and analyzed after 14 days incubation. $^*P<0.05$ or $^{**}P<0.01$ by analysis of variance test. (**d**) U937, HL-60, NB4 cells and immortalized B cells were treated with anti-IGF-IR (1 μg/ml) or anti-IR (5 μg/ml) neutralizing antibodies and proliferation was measured by MTS assay. Cell proliferation rate was expressed as percentage of cells treated with a control antibody (CTR, OKT3). LY294002 was used as a control. (**e**) AML patient blasts (1; FAB M0) were incubated with increasing concentrations of NVP-AEW541 and analyzed for caspase-3 activity and PARP cleavage. The band corresponding to uncleaved PARP (116 kDa) is shown. (**f**) NB4 (A) and U937 (B) cells were incubated with increasing concentrations of NVP-AEW541 and DNA fragmentation was analyzed by detection of cells with fractional (Sub-G_1) DNA content using propidium iodide (PI) staining and FACS analysis.

(10 μM) significantly decreased the colony-forming ability (20% reduction), low concentrations (1–5 μM) of the inhibitor did not significantly affect the response (Figure 4c).

To confirm the data obtained with the IGF-IR kinase inhibitor, a neutralizing antibody directed against the receptor was tested for its ability to inhibit AML cell proliferation. Three AML cell lines (U937, HL-60 and NB4) were analyzed. A significant inhibition of cell proliferation was observed when all three AML cell lines were treated with the IGF-IR neutralizing antibody, as compared to a control (OKT3) antibody or immortalized B cells (Figure 4d). Similar results were obtained with a neutralizing antibody against the IR in U937 and HL-60 cells, but not in NB4, which express low levels of the receptor (Figure 1a).

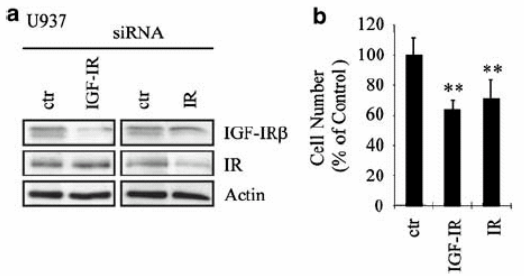

Figure 5 Inhibition of AML cell proliferation by small interfering RNA (siRNA) targeting the insulin-like growth factor I (IGF-IR) or insulin receptor (IR). (**a**) U937 cells transfected with control siRNA or siRNA targeting the IGF-IR or the IR were analyzed by western blotting for the expression of the receptors. (**b**) Cell proliferation of U937 cells transfected with siRNA targeting the IGF-IR or IR was analyzed by MTS assay. $^{**}P<0.01$ by analysis of variance test.

The ability of the IGF-IR kinase inhibitor to induce apoptosis was then investigated in AML blasts and cell lines. Treatment of purified AML blasts with increasing concentrations of NVP-AEW541 resulted in increased activation of caspase-3 and enhanced cleavage of PARP (Figure 4e), demonstrating the induction of apoptosis in these cells. Consistently, NVP-AEW541-induced cell death was observed in NB4 and U937 cells, as assessed by propidium iodide (PI) staining and fluorescence-activated cell sorting analysis (Figure 4f). Reduced cell proliferation was also observed when U937 cells were transfected with siRNA specifically targeting the IGF-IR or IR (Figures 5a and b), further confirming the results obtained with NVP-AEW541 or neutralizing antibodies.

Inhibition of the IGF-IR kinase activity enhances the effects of apoptosis in AML blasts and cell lines induced by etoposide or Ara-C

AML treatment is frequently complicated by resistance to chemotherapy. On that account, we investigated whether inhibition of IGF-IR signaling by NVP-AEW541 could sensitize human AML cells to the action of chemotherapeutic agents. Increasing concentrations of etoposide or Ara-C resulted in effective suppression of cell growth in U937, THP1, HL-60 and NB4 AML cell lines with IC_{50} values in the range of 1.0–5.0 μM and 0.1–2.0 μM, respectively (data not shown). AML cells were then exposed to etoposide or Ara-C (1.0 μM each) in the presence of increasing concentrations of NVP-AEW541. Combination treatment enhanced the effect of IGF-IR inhibition in NVP-AEW541-sensitive cells (Figure 6a), whereas the effect was negligible in cells with lower responsiveness to the inhibitor (Figure 6b). Already at a low concentration of etoposide (1.0 μM), nontoxic concentrations of NVP-AEW541 increased the effect of the chemotherapeutic drug on NB4 cells

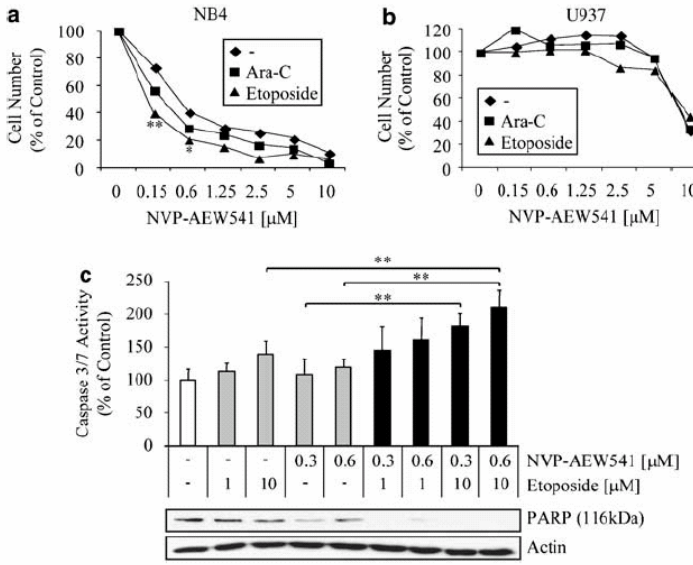

Figure 6 NVP-AEW541 sensitizes AML cells to chemotherapeutical agents. Cell proliferation rate of (**a**) NB4 or (**b**) U937 cells incubated with increasing concentrations of NVP-AEW541 in the absence or presence of cytarabine (Ara-C) or etoposide (1.0 μM each). A representative experiment (out of three) performed with eight repetitions is shown. $^{*}P<0.05$ or $^{**}P<0.01$ by analysis of variance test. (**c**) AML patient blasts (13; FAB M5) were treated with different concentrations of NVP-AEW541 (0.3, 0.6 μM) in combination with different concentrations of etoposide (1.0, 10 μM). Cell lysates were analyzed for caspase-3 activity and evaluated by western blotting for PARP cleavage. The band corresponding to uncleaved PARP (116 kDa) is shown.

(Figure 6a). Furthermore, combination treatment of etoposide and NVP-AEW541 elevated caspase-3 activity in primary AML blasts and induced PARP cleavage (Figure 6c), demonstrating enhanced apoptosis. Thus, pharmacological IGF-IR kinase inhibitors sensitize human AML cells to etoposide.

Discussion

Pharmacological inhibitors targeting the tyrosine kinase activity of receptor tyrosine kinase (RTKs) have been extensively studied as antitumor agents, such as in the case of the epidermal growth factor receptor in solid tumors and FLT3 inhibitors in leukemia.[9,36] Differences in the sensitivities of human tumor cells to such agents can be caused by mutations in the receptor.[37] Thus, in leukemia, FLT3 mutations were reported to correlate with sensitivity of tumor cells to RTK inhibitors.[38] Moreover, FLT3 mutations were recently shown to correlate with decreased patient survival in AML patients.[39] Although activating mutations have not been described in the IGF-IR in most human cancers, the receptor is frequently activated by overexpression and/or establishment of autocrine loops involving the ligands IGF-I and IGF-II.[12,40] Previous reports have investigated the expression of the IGF-IR in human AML cells and described a role for IGF-I as a growth factor for these cells in combination with other cytokines.[13,41] Increased serum levels of the IGFBP-2 have also been observed in patients with AML.[42]

In the present study, we have further investigated the biological role of IGF-IR signaling in human AML blasts and cell lines. For the first time, we show that IGF-I promotes growth of AML blasts and activates intracellular signaling mediators including the PI3K/Akt and Erk pathways. Expression of the IGF-IR was found in a panel of primary AML blasts and cell lines, and IGF-I was consistently secreted by blasts and cell lines, implying that an autocrine signaling loop involving IGF-I and the IGF-IR is present in AML. Indeed, the levels of IGF-I detected in the supernatant of AML cells were shown to activate intracellular signaling events in the cells, implying that they are biologically relevant. The autocrine signaling loop involving IGF-I may contribute to the constitutive activation of Akt observed in AML blasts and cell lines. Moreover, the related IR was also found overexpressed in AML blasts, as compared to nonleukemic bone marrow cells, and insulin was able to activate PI3K/Akt and Erk in a manner comparable to IGF-I. Thus, both IGF-I and insulin may represent novel growth factors playing a crucial role in AML cell proliferation *in vivo*.

The IGF-IR kinase inhibitor NVP-AEW541 was able to reduce growth of AML blasts and cell lines, although differences in sensitivities were observed. In the case of some AML cell lines, high levels of expression of the IR were found in cells with reduced sensitivities to NVP-AEW541, which is in agreement with the observation that the inhibitor also blocks the protein tyrosine kinase activity of the IR at higher concentrations than the IGF-IR.[30] In support of this notion, both IGF-IR and IR neutralizing antibodies partially reduced the growth of AML cell lines. However, additional molecular mechanisms may render some AML cell lines or primary blasts resistant to IGF-IR inhibitors. Indeed, autocrine stem cell factor/c-Kit signaling was shown to reduce the sensitivity of human small cell lung cancer cells to the related IGF-IR kinase inhibitor NVP-ADW742.[43] Moreover, enhanced activation of Akt was also recently shown to protect human neuroblastoma cells from the effects of NVP-AEW541.[33] Finally, it was shown that, in hematopoietic cells transfected with the IGF-IR, pharmacological inhibitors of PI3K/Akt/mTOR and the Raf/MEK/Erk pathways enhanced the effects of IGF-IR neutralizing antibodies.[44] Thus, a variety of mechanisms could explain the differences in sensitivities to NVP-AEW541 observed in AML blasts and cell lines. The ability of NVP-AEW541 to suppress the growth of AML cells was in part the result of induction of apoptosis, suggesting that the IGF-IR plays a role in survival of AML cells. The IGF-IR kinase inhibitor also sensitized AML cells to the chemotherapeutic agent etoposide, an observation that was previously made in other cancer cell lines.[45] Thus, IGF-IR signaling also plays a role in the resistance of AML cells to chemotherapeutic agents. Nonleukemic bone marrow cells, normal $CD34^+$ hematopoietic progenitors and immortalized B lymphocytes were resistant to pharmacological IGF-IR inhibitors and neutralizing antibodies, indicating that targeting IGF-IR signaling may result in selective killing of AML cells, as compared to normal leukocytes *in vivo*.

PI3K signaling was demonstrated to contribute to AML cell growth and survival,[46] and recent reports showed that the class I_A PI3K isoform p110δ plays a major role in AML cell proliferation.[47,48] By using RNAi, we show for the first time that in addition to p110δ, the PI3K isoform p110β also plays a role in AML cell growth and survival. These observations were supported by the broad expression of these two isoforms in AML blasts and cell lines. Indeed, this is the first study showing that p110β is overexpressed in most AML cases. The overexpression of p110δ in AML blasts and cell lines investigated here is consistent with previous studies.[47,48] In contrast, only low levels of p110α mRNA and protein could be found in the subset of patient samples and AML cell lines analyzed in this study. These differences could be caused by the heterogeneity and relatively small size of the samples analyzed in each study. Interestingly, RNAi or pharmacological inhibitors targeting p110β and p110δ reduced Akt activation by IGF-I or insulin, implying that both PI3K isoforms can couple to the activated IGF-IR or IR in AML cells. Recent reports have demonstrated a selective role for p110α in transducing signals from the IR that control glucose homeostasis in insulin-sensitive tissues.[49,50] However, previous reports have also documented a role for p110β in transducing signals from the IR controlling cytoskeletal rearrangements.[51] The most likely explanation for these apparent discrepancies are differences in PI3K isoform expression between the tissues/cell systems studied, since expression of p110α was detected only weakly in a subset of the AML cell lines and blasts studied here, and insulin-sensitive tissues may not express p110δ. Reports concerning the expression of the PI3K antagonist PTEN in AML blasts are controversial. While variable or nondetectable PTEN expression levels have been reported in individual AML cases,[27] a subsequent study found PTEN expression in all AML cases investigated.[28] The latter findings are in line with the results obtained in the present study.

Together our data document a novel role for autocrine IGF-I/IGF-IR signaling in the biology of human AML, which may provide novel therapeutic targets to inhibit the proliferation of leukemic cells.

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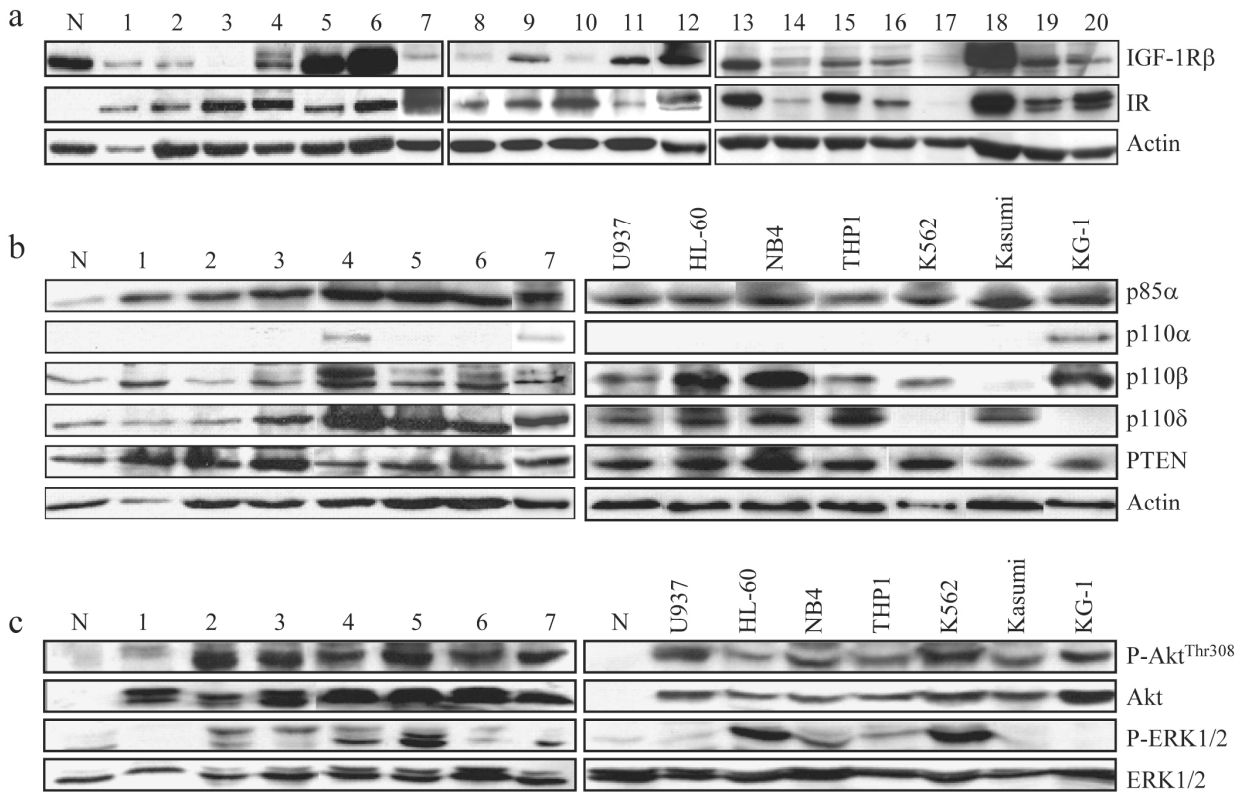
References

1 Kelly LM, Gilliland DG. Genetics of myeloid leukemias. *Annu Rev Genomics Hum Genet* 2002; **3**: 179–198.
2 Meshinchi S, Smith FO, Arceci RJ. Prognostic factors and risk-based therapy in pediatric acute myeloid leukemia. *Curr Oncol Rep* 2003; **5**: 489–497.
3 Bieker R, Padro T, Kramer J, Steins M, Kessler T, Retzlaff S *et al.* Overexpression of basic fibroblast growth factor and autocrine stimulation in acute myeloid leukemia. *Cancer Res* 2003; **63**: 7241–7246.
4 Fiedler W, Graeven U, Ergun S, Verago S, Kilic N, Stockschlader M *et al.* Vascular endothelial growth factor, a possible paracrine growth factor in human acute myeloid leukemia. *Blood* 1997; **89**: 1870–1875.
5 Zheng R, Levis M, Piloto O, Brown P, Baldwin BR, Gorin NC *et al.* FLT3 ligand causes autocrine signaling in acute myeloid leukemia cells. *Blood* 2004; **103**: 267–274.
6 Gilliland DG, Griffin JD. The roles of FLT3 in hematopoiesis and leukemia. *Blood* 2002; **100**: 1532–1542.
7 Ikeda H, Kanakura Y, Tamaki T, Kuriu A, Kitayama H, Ishikawa J *et al.* Expression and functional role of the proto-oncogene c-kit in acute myeloblastic leukemia cells. *Blood* 1991; **78**: 2962–2968.
8 Aguayo A, Estey E, Kantarjian H, Mansouri T, Gidel C, Keating M *et al.* Cellular vascular endothelial growth factor is a predictor of outcome in patients with acute myeloid leukemia. *Blood* 1999; **94**: 3717–3721.
9 Tse KF, Allebach J, Levis M, Smith BD, Bohmer FD, Small D. Inhibition of the transforming activity of FLT3 internal tandem duplication mutants from AML patients by a tyrosine kinase inhibitor. *Leukemia* 2002; **16**: 2027–2036.
10 O'Farrell AM, Abrams TJ, Yuen HA, Ngai TJ, Louie SG, Yee KW *et al.* SU11248 is a novel FLT3 tyrosine kinase inhibitor with potent activity *in vitro* and *in vivo*. *Blood* 2003; **101**: 3597–3605.
11 Brown P, Small D. FLT3 inhibitors: a paradigm for the development of targeted therapeutics for paediatric cancer. *Eur J Cancer* 2004; **40**: 707–721; discussion 22–4.
12 Khandwala HM, McCutcheon IE, Flyvbjerg A, Friend KE. The effects of insulin-like growth factors on tumorigenesis and neoplastic growth. *Endocr Rev* 2000; **21**: 215–244.
13 Hizuka N, Sukegawa I, Takano K, Asakawa K, Horikawa R, Tsushima T *et al.* Characterization of insulin-like growth factor I receptors on human erythroleukemia cell line (K-562 cells). *Endocrinol Jpn* 1987; **34**: 81–88.
14 Sukegawa I, Hizuka N, Takano K, Asakawa K, Shizume K. Decrease in IGF-I binding sites on human promyelocytic leukemia cell line (HL-60) with differentiation. *Endocrinol Jpn* 1987; **34**: 365–372.
15 Neri LM, Borgatti P, Tazzari PL, Bortul R, Cappellini A, Tabellini G *et al.* The phosphoinositide 3-kinase/AKT1 pathway involvement in drug and all-trans-retinoic acid resistance of leukemia cells. *Mol Cancer Res* 2003; **1**: 234–246.
16 Yasui H, Hideshima T, Richardson PG, Anderson KC. Novel therapeutic strategies targeting growth factor signalling cascades in multiple myeloma. *Br J Haematol* 2006; **132**: 385–397.
17 Mitsiades CS, Mitsiades NS, McMullan CJ, Poulaki V, Shringarpure R, Akiyama M *et al.* Inhibition of the insulin-like growth factor receptor-1 tyrosine kinase activity as a therapeutic strategy for multiple myeloma, other hematologic malignancies, and solid tumors. *Cancer Cell* 2004; **5**: 221–230.
18 Yamamoto K, Altschuler D, Wood E, Horlick K, Jacobs S, Lapetina EG. Association of phosphorylated insulin-like growth factor-I receptor with the SH2 domains of phosphatidylinositol 3-kinase p85. *J Biol Chem* 1992; **267**: 11337–11343.
19 Vanhaesebroeck B, Leevers SJ, Panayotou G, Waterfield MD. Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem Sci* 1997; **22**: 267–272.
20 Katso R, Okkenhaug K, Ahmadi K, White S, Timms J, Waterfield MD. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Annu Rev Cell Dev Biol* 2001; **17**: 615–675.
21 Aggerholm A, Gronbaek K, Guldberg P, Hokland P. Mutational analysis of the tumour suppressor gene MMAC1/PTEN in malignant myeloid disorders. *Eur J Haematol* 2000; **65**: 109–113.
22 Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* 1998; **273**: 13375–13378.
23 Kang S, Bader AG, Vogt PK. Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic. *Proc Natl Acad Sci USA* 2005; **102**: 802–807.
24 Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S *et al.* High frequency of mutations of the PIK3CA gene in human cancers. *Science* 2004; **304**: 554.
25 Liu TC, Lin PM, Chang JG, Lee JP, Chen TP, Lin SF. Mutation analysis of PTEN/MMAC1 in acute myeloid leukemia. *Am J Hematol* 2000; **63**: 170–175.
26 Hummerdal P, Andersson P, Willander K, Linderholm M, Soderkvist P, Jonsson JI. Absence of hot spot mutations of the PIK3CA gene in acute myeloid leukaemia. *Eur J Haematol* 2006; **77**: 86–87.
27 Xu Q, Simpson SE, Scialla TJ, Bagg A, Carroll M. Survival of acute myeloid leukemia cells requires PI3 kinase activation. *Blood* 2003; **102**: 972–980.
28 Grandage VL, Gale RE, Linch DC, Khwaja A. PI3-kinase/Akt is constitutively active in primary acute myeloid leukaemia cells and regulates survival and chemoresistance via NF-kappaB, Mapkinase and p53 pathways. *Leukemia* 2005; **19**: 586–594.
29 Fukuda R, Hayashi A, Utsunomiya A, Nukada Y, Fukui R, Itoh K *et al.* Alteration of phosphatidylinositol 3-kinase cascade in the multilobulated nuclear formation of adult T cell leukemia/lymphoma (ATLL). *Proc Natl Acad Sci USA* 2005; **102**: 15213–15218.
30 Garcia-Echeverria C, Pearson MA, Marti A, Meyer T, Mestan J, Zimmermann J *et al.* *In vivo* antitumor activity of NVP-AEW541-A novel, potent, and selective inhibitor of the IGF-IR kinase. *Cancer Cell* 2004; **5**: 231–239.
31 Marra G, D'Atri S, Corti C, Bonmassar L, Cattaruzza MS, Schweizer P *et al.* Tolerance of human MSH2+/− lymphoblastoid cells to the methylating agent temozolomide. *Proc Natl Acad Sci USA* 2001; **98**: 7164–7169.
32 Berns K, Hijmans EM, Mullenders J, Brummelkamp TR, Velds A, Heimerikx M *et al.* A large-scale RNAi screen in human cells identifies new components of the p53 pathway. *Nature* 2004; **428**: 431–437.
33 Guerreiro AS, Boller D, Shalaby T, Grotzer MA, Arcaro A. Protein kinase B modulates the sensitivity of human neuroblastoma cells to insulin-like growth factor receptor inhibition. *Int J Cancer* 2006; **119**: 2527–2538.
34 Jackson SP, Schoenwaelder SM, Goncalves I, Nesbitt WS, Yap CL, Wright CE *et al.* PI 3-kinase p110beta: a new target for antithrombotic therapy. *Nat Med* 2005; **11**: 507–514.
35 Sadhu C, Masinovsky B, Dick K, Sowell CG, Staunton DE. Essential role of phosphoinositide 3-kinase delta in neutrophil directional movement. *J Immunol* 2003; **170**: 2647–2654.
36 Ciardiello F, Caputo R, Bianco R, Damiano V, Pomatico G, De Placido S *et al.* Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. *Clin Cancer Res* 2000; **6**: 2053–2063.
37 Grundler R, Thiede C, Miething C, Steudel C, Peschel C, Duyster J. Sensitivity toward tyrosine kinase inhibitors varies between different activating mutations of the FLT3 receptor. *Blood* 2003; **102**: 646–651.
38 Stone RM, DeAngelo DJ, Klimek V, Galinsky I, Estey E, Nimer SD *et al.* Patients with acute myeloid leukemia and an activating mutation in FLT3 respond to a small-molecule FLT3 tyrosine kinase inhibitor, PKC412. *Blood* 2005; **105**: 54–60.
39 Thiede C, Steudel C, Mohr B, Schaich M, Schakel U, Platzbecker U *et al.* Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. *Blood* 2002; **99**: 4326–4335.
40 Nakanishi Y, Mulshine JL, Kasprzyk PG, Natale RB, Maneckjee R, Avis I *et al.* Insulin-like growth factor-I can mediate autocrine proliferation of human small cell lung cancer cell lines *in vitro*. *J Clin Invest* 1988; **82**: 354–359.
41 Shimon I, Shpilberg O. The insulin-like growth factor system in regulation of normal and malignant hematopoiesis. *Leuk Res* 1995; **19**: 233–240.

42 Dawczynski K, Kauf E, Zintl F. Changes of serum growth factors (IGF-I, -II and IGFBP-2, -3) prior to and after stem cell transplantation in children with acute leukemia. *Bone Marrow Transplant* 2003; **32**: 411–415.
43 Warshamana-Greene GS, Litz J, Buchdunger E, Hofmann F, Garcia-Echeverria C, Krystal GW. The insulin-like growth factor-I (IGF-I) receptor kinase inhibitor NVP-ADW742, in combination with STI571, delineates a spectrum of dependence of small cell lung cancer on IGF-I and stem cell factor signaling. *Mol Cancer Ther* 2004; **3**: 527–535.
44 Bertrand FE, Steelman LS, Chappell WH, Abrams SL, Shelton JG, White ER *et al*. Synergy between an IGF-1R antibody and Raf/MEK/ERK and PI3K/Akt/mTOR pathway inhibitors in suppressing IGF-1R-mediated growth in hematopoietic cells. *Leukemia* 2006; **20**: 1254–1260.
45 Warshamana-Greene GS, Litz J, Buchdunger E, Garcia-Echeverria C, Hofmann F, Krystal GW. The insulin-like growth factor-I receptor kinase inhibitor, NVP-ADW742, sensitizes small cell lung cancer cell lines to the effects of chemotherapy. *Clin Cancer Res* 2005; **11**: 1563–1571.
46 Martelli AM, Nyakern M, Tabellini G, Bortul R, Tazzari PL, Evangelisti C *et al*. Phosphoinositide 3-kinase/Akt signaling pathway and its therapeutical implications for human acute myeloid leukemia. *Leukemia* 2006; **20**: 911–928.
47 Sujobert P, Bardet V, Cornillet-Lefebvre P, Hayflick JS, Prie N, Verdier F *et al*. Essential role for the p110delta isoform in phosphoinositide 3-kinase activation and cell proliferation in acute myeloid leukemia. *Blood* 2005; **106**: 1063–1066.
48 Billottet C, Grandage VL, Gale RE, Quattropani A, Rommel C, Vanhaesebroeck B *et al*. A selective inhibitor of the p110delta isoform of PI 3-kinase inhibits AML cell proliferation and survival and increases the cytotoxic effects of VP16. *Oncogene* 2006; **25**: 6648–6659.
49 Foukas LC, Claret M, Pearce W, Okkenhaug K, Meek S, Peskett E *et al*. Critical role for the p110alpha phosphoinositide-3-OH kinase in growth and metabolic regulation. *Nature* 2006; **441**: 366–370.
50 Knight ZA, Gonzalez B, Feldman ME, Zunder ER, Goldenberg DD, Williams O *et al*. A pharmacological map of the PI3-K family defines a role for p110alpha in insulin signaling. *Cell* 2006; **125**: 733–747.
51 Hooshmand-Rad R, Hajkova L, Klint P, Karlsson R, Vanhaesebroeck B, Claesson-Welsh L *et al*. The PI 3-kinase isoforms p110(alpha) and p110(beta) have differential roles in PDGF- and insulin-mediated signaling. *J Cell Sci* 2000; **113** (Part 2): 207–214.

Supplementary information accompanies the paper on the Leukemia web site (http://www.nature.com/leu)

Supplementary Figure 1



Supplementary Figure 1 (**a**) Expression of IGF-IRβ and IR in 20 human AML patient blast cells and normal bone marrow cells (N). (**b**) PI3K class I_A regulatory subunit p85α, catalytic subunits p110α, p110β, p110δ and the PI3K antagonist phosphatase and tensin homolog (PTEN). (**c**) Pathway activation monitored by the phosphorylation status of protein kinase B (PKB/Akt: Thr308) and extracellular signal-regulated kinase (Erk: Thr202/Tyr204). Actin was used as a loading control.

Supplementary Table 1

French-American-British (FAB) classification of the AML patient blast cells under study.

Patient	FAB
1	M0
2	M0
3	M0
4	M4
5	M1
6	M4
7	M1
8	M2
9	M4
10	M5
11	M2
12	M5
13	M5
14	M1
15	M1
16	M4
17	M2
18	M5
19	M2
20	M2

3.3. Targeting PI3KC2β impairs proliferation and survival in acute leukemia, brain tumours and neuroendocrine tumours
(British Journal of Cancer; Manuscript submitted)

Targeting PI3KC2β impairs proliferation and survival in acute leukemia, brain tumours and neuroendocrine tumours

Running Title: PI3KC2β in human cancers

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ABSTRACT

Eight mammalian phosphoinositide 3-kinase (PI3K) isoforms exist which are subdivided into three classes. While much attention has been given to the class I isoforms, little is known about the functions of class II PI3Ks in human cancer. The expression pattern and functions of the PI3KC2β isoform were investigated in a panel of tumour samples and cell lines. Over-expression of PI3KC2β was found in subsets of tumours and cell lines from acute myeloid leukemia (AML), glioblastoma multiforme (GBM), medulloblastoma (MB), neuroblastoma (NB), and small cell lung cancer (SCLC). Specific pharmacological inhibitors of PI3KC2β or small interfering RNA (siRNA) impaired proliferation of a panel of cell lines and primary cultures from AML, brain tumours and neuroendocrine tumours. Inhibition of PI3KC2β also induced apoptosis in AML and GBM cell lines and sensitised the cells to chemotherapeutic agents. Furthermore, PI3KC2β inhibition impaired the phosphorylation of downstream signalling mediators in AML. Together, these data show that PI3KC2β contributes to proliferation and survival in AML, brain tumours and neuroendocrine tumours and may represent a novel target in these malignancies.

Keywords: PI3KC2β, pharmacological inhibition, cell proliferation, migration

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INTRODUCTION

Phosphoinositide 3-kinases (PI3Ks) play an essential role in the signal transduction events initiated by the binding of extracellular signals to their cell surface receptors (Katso et al., 2001; Vanhaesebroeck et al., 2001). The cellular responses controlled by PI3Ks are extremely diverse, including mitogenesis and proliferation, protection from apoptosis and cell motility (Katso et al., 2001; Vanhaesebroeck et al., 2001). There are eight known PI3Ks in humans, which have been subdivided into three classes, based on structural homology and *in vitro* substrate specificity (Vanhaesebroeck et al., 1997a; Vanhaesebroeck & Waterfield, 1999). Class I_A comprises three highly homologous isoforms, p110α (Hiles et al., 1992), p110β (Hu et al., 1993) and p110δ (Chantry et al., 1997; Vanhaesebroeck et al., 1997b), which exist as a heterodimeric complex with a regulatory subunit containing two Src homology-2 (SH2) domains, mediating enzyme association with phosphotyrosine residues in the cytoplasmic domains of activated polypeptide growth factor receptors (Inukai et al., 1996; Otsu et al., 1991; Pons et al., 1995). All class I PI3Ks function as PtdIns(4,5)P_2 3-kinase *in vivo*, upon activation by receptor tyrosine kinases or serpentine receptors (Hawkins et al., 1992; Stephens et al., 1991). PtdIns(3,4,5)P_3 serves as a docking site for the serine/threonine protein kinase phosphoinositide-dependent protein kinase-1 (PDK-1) which is activated upon binding (Alessi et al., 1997). Several protein kinases have been identified as downstream targets of PDK-1, such as the serine/threonine protein kinase B (PKB) /Akt, which is a key regulator of cell survival, ribosomal protein S6 kinase (S6K), which stimulates protein synthesis and cell growth, glycogen synthase kinase-3 (GSK-3), a key regulator of glycogen synthesis, and a subset of protein kinase C (PKC) isoforms (Le Good et al., 1998; Vanhaesebroeck & Alessi, 2000).

Class II PI3Ks comprise the Drosophila PI3K_68D/Cpk (MacDougall et al., 1995; Molz et al., 1996), mouse Cpk-m (Molz et al., 1996), and human PI3KC2α (Domin et al., 1997), PI3KC2β (Arcaro et al., 1998) and PI3KC2γ (Misawa et al., 1998; Ono et al., 1998). The hallmarks of class II family members are a substrate specificity restricted to PtdIns and PtdIns(4)P *in vitro*, and a conserved C-terminal C2 domain, involved in phospholipid binding. Recent studies have started to investigate the regulation and functions of class II PI3Ks *in vivo*. Indeed, several reports have shown that class II PI3Ks are downstream targets of activated receptor tyrosine kinases (RTKs), such as the epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), c-Kit and insulin receptor (IR) (Arcaro et al., 2002; Arcaro et al., 2000; Brown et al., 1999; Katso et al., 2006). Studies in *Drosophila melanogaster* have also

revealed a role for the class II PI3K_68D in cell differentiation downstream of the EGFR (MacDougall et al., 2004). Furthermore, recent reports have documented a role for PI3KC2β in cell migration in mammalian cells via activation of Rho family GTPases and in human cancer cells (Domin et al., 2005; Katso et al., 2006; Maffucci et al., 2005). Studies by others have described a role for PI3KC2α in Rho activation and contraction in vascular smooth muscle cells (Wang et al., 2006). Analysis of a conditional knock-out mouse of *PIK3C2B*, however, revealed no obvious phenotype despite a fairly ubiquitous deletion of *PIK3C2B* (Harada et al., 2005).

The importance of PI3K signalling in cancer is highlighted by the fact that mutations in the tumour suppressor gene phosphatase and tensin homologue (*PTEN*) occur frequently in human tumors. PTEN is a phosphatase that antagonises the action of PI3K by de-phosphorylating the D-3 position of polyphosphoinositides (Cantley & Neel, 1999; Sansal & Sellers, 2004). Moreover, recent reports have described activating mutations in the *PIK3CA* gene encoding the catalytic p110α isoform of PI3K in a variety of human cancers including breast, colon and ovarian cancers (Broderick et al., 2004; Samuels et al., 2004; Weir et al., 2004).

In the present report we have evaluated the expression of the class II PI3KC2β isoform in a panel of primary human tumours and cell lines. Furthermore, we have used isoform-specific pharmacological inhibitors and RNAi to inhibit this enzyme in human cancer cell lines. We show for the first time that PI3KC2β is over-expressed in acute myeloid leukemia, brain tumours and neuroendocrine tumours and that inhibiting this class II PI3K decreases proliferation and survival in cell lines from these cancers.

MATERIALS AND METHODS

Reagents and antibodies

Antibodies and reagents were purchased from the following companies: The PI3KC2β antibody was described in (Arcaro et al., 1998). Caspase-3, PARP (Santa Cruz Biotechnology, Santa Cruz, CA, USA); activated Akt/PKB (Ser473), activated JNK (Thr183/Tyr185), activated S6 protein (Ser235/236) (Cell Signalling Technology, Danvers, MA, USA); β-actin, β-tubulin (Sigma-Aldrich, St Louis, MO, USA); siGENOME™ siRNA (Dharmacon, Lafayette, CO, USA);. (PIramed, Berkshire, UK); Etoposide (Calbiochem, La Jolla, CA, USA); Doxorubicin (Pfizer AG, Zurich, CH). PI701 (YM185453) and PI702 (YM182832) were provided by Piramed Pharma Limited and their synthetic details will be the subject of a separate publication.

Apoptosis

For detection of apoptosis, cells were incubated for 16-24 hrs in the presence or absence of inhibitors. The cells were lysed and caspase-3 activity was measured using the CaspACE Assay System (Promega). Additionally, samples were analysed by SDS-PAGE and Western blot with anti-caspase-3 or anti-poly(ADP-ribose) polymerase (PARP) antibodies.

Cell culture

Acute myeloid leukemia, neuroblastoma, glioblastoma, medulloblastoma, small cell lung cancer and mammary epithelial cell lines were grown in RPMI or DMEM (Life Technologies/InvitrogenCarlsbad, CA, USA) with 10% (v/v) fetal calf serum (FCS) and penicillin/streptomycin/L-glutamine, and passaged every 3-5 days. DAOY, D341, D425 and D458 medulloblastoma cell lines were grown in MEM Zinc option (Richter's modification) medium supplemented with 10% FCS. For growth factor stimulations cells were incubated overnight in their growth medium with low serum (0.5-1% v/v) or Optimem medium (Life Technologies/Invitrogen) and washed with serum-free medium prior to incubation with growth factors. Heparinized peripheral blood or bone marrow samples were obtained from adult patients with AML. Blast cells were isolated as described previously (Doepfner et al., 2007). Type II human lung pneumocytes (Pardo et al., 2001) were maintained in DCCM-1 medium supplemented with 10% new born calf serum.

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Cell proliferation

Cell lines (5 x 10^3 cells/well) were seeded in 96-well plates and grown for 72h in serum (10%)-containing medium in the presence or absence of inhibitors. The number of viable cells was analysed by means of an MTS assay using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Data are mean with SD from 8 repetitions.

Dissociation of brain tumours

Human brain tumours were removed from 4 patients who underwent surgery for tumour resection at the University Hospital Zurich. The procedures were conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the Canton Zurich. Following removal, tumour tissue was immediately placed in a petri dish, minced mechanically and digested enzymatically with collagenase D and DNase I (Roche Applied Science, Rotkreuz, Switzerland) for 1 hour at 37°C while being stirred with a magnetic bar. The dissociated cells were then sequentially filtered through 100 and 70μm cell strainers (BD Falcon, BD Biosciences, Basel, Switzerland) to remove any tissue debris. Erythrocytes were removed by resuspending and incubating the cells in ice-cold ACK buffer (17 mM Tris–HCl [pH 7.2] containing 144 mM NH4Cl) for 10 minutes on ice. The cells were washed in PBS and plated in DMEM (Life Technologies/Invitrogen) supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS) and gentamycin (20mg/ml) and passaged every 3-5 days by trypsinization.

DNA Microarray

Total RNA was extracted from 60 medulloblastoma samples (MB) and 3 cell lines (D283, D341, and DAOY) using the Trizol reagent (Invitrogen). After DNAse treatment and RNA purification (RNeasy Micro kit, Qiagen), gene expression profiles were obtained on the Affymetrix HG-U133 Plus 2.0 array that contains more than 54000 probe sets for transcripts and variants. Expression data for 9 normal cerebellums analyzed on the same Affymetrix array version were obtained from Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). Gene expression data for the samples were normalized using the GCRMA procedure. PI3KC2β expression levels for the samples are presented (unlogged data).

Isolation of RNA from tumour samples and RT-PCR

For primary neuroblastoma samples ethical approval to use residual tissue was obtained. RNAlater-preserved tumour tissue was available in the Swiss Pediatric Oncology Group tumour bank. All diagnoses were confirmed by histological assessment of the tumour specimen obtained at surgery. Neuroblastoma tissue was disrupted with a sterile disposable tissue grinder (Sage Products, Cary, IL, USA) and homogenised in guanidinium isothiocyanate-containing buffer. Total RNA of cell lines or tumour tissue was isolated using the RNeasy kit (Qiagen, Santa Cruz, CA, USA) according to the manufacturer's protocol. Total RNA (3 μg) from each tumour sample was converted into cDNA using the SuperScript™ First-Strand Synthesis System for PCR according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). mRNA expression of PI3KC2β and 18S (internal control gene) was measured in tumour samples and cell lines by TaqMan® Assay-on-Demand™ Gene Expression products (Applied Biosystems, Foster City, CA, USA). The following primers were used (gene - assay ID): PI3KC2beta - Hm00153248_m1; eukaryotic 18S rRNA - Hs99999901_s1. Three replicates were run for each sample in a 96-well format plate. Gene expression assays consisted of a FAM™ dye-labelled TaqMan® MGB probe and two PCR primers. The thermal cycling conditions consisted of an initial denaturation step at 95°C for 10 min and a 50-cycle countdown at 95°C for 15 s and 60°C for 1 min. Each sample was normalised on the basis of its 18S rRNA content. Relative mRNA expression levels were calculated using the comparative threshold cycle (CT) method (Giulietti et al 2001).

PI3K Assays

Recombinant human PI3KC2β was expressed as a glutathione *S*-transferase (GST)-fusion protein in SF9 insect cells and purified as described previously (Arcaro et al., 1998). Recombinant class I PI3K isoforms were expressed and purified likewise. PI3K activity of the different PI3K isoforms was assayed essentially as described (Hayakawa et al., 2007).

SDS-PAGE and Western blot analysis

Cellular lysates were prepared as previously described (Guerreiro et al., 2006) separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a hydrophobic polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham Biosciences), and immunoblotted with various antibodies according to the manufacturer's protocol. Chemiluminescence was used for visualization using the enhanced

chemiluminescence (ECL) Western blotting detection reagents (Amersham Biosciences) according to the manufacturer's protocol.

Transient expression in AML cells

AML cells were transfected with small interfering RNA (siRNA) targeting PI3KC2β using the Amaxa Nucleofector system (Amaxa biosystems, Gaithersburg, MD, USA) according to the manufacturer's protocol. Cell Line Nucleofector Kit V was used and program V-001 applied. After 48h cells were lysed in cell lysis buffer in order to visualize protein expression by SDS-PAGE and Western blotting. Besides, cells were analysed for cell proliferation and apoptosis by MTS assay and Caspase-3 measurement 72h after transfection.

Wound healing assay

EpH4 murine epithelial cells stably transfected with the Ha-*Ras* oncogene and induced by TGF-β1 to undergo epitheliomesenchymal transition (EMT) *in vivo* to establish FibRas cells were previously described in (Maschler et al., 2005). For wound healing assays, cells were plated in 12-well culture plates in complete medium and grown to confluency. A wound was created by scraping cells with a 200µl tip. The migration rate was monitored for eight hours by phase contrast microscopy (Leica DM IRBE Inverse, Widefield) either in the presence or absence of inhibitors.

RESULTS

Expression of PI3KC2β in tumour samples and cell lines

Previous reports had documented increased expression of PI3KC2β in leukemia, glioblastoma and lung cancer cell lines and tumours (Arcaro et al., 2002; Knobbe & Reifenberger, 2003; Qian et al., 2002). Therefore, the expression of PI3KC2β was investigated in a panel of tumours and cell lines from AML, neuroendocrine tumours (SCLC and NB) and brain tumours (MB and GBM). In AML, PI3KC2β was highly expressed in a subset of AML blasts and cell lines (Figure 1A, left panel). Interestingly, PI3KC2β expression was much lower in non-leukemic bone marrow cells and immortalised B cells (Figure 1A) indicating that AML blasts and cell lines over-express the enzyme. We also reanalysed cDNA microarray data from a previously published study in AML (Valk et al., 2004). PI3KC2β mRNA expression was found to be higher in certain groups of AML, depending on molecular and cytogenetic abnormalities. AML categories displaying increased expression included FLT3-ITD and EVI1 (77.37; 99.21), as compared to FLT3-TKD (41.51). Increased expression of PI3KC2β was also found in AML with the cytogenetic abnormalities -7 (99.72), when compared to +8, 11q13, t(8;21), idt(16), or NN (46.86; 39.35; 54.40; 19.80; 60.03). In contrast, there were no significant differences in expression of PI3KC2β mRNA between AML French-American-British (FAB) classes.

Protein expression analysis of SCLC cell lines revealed elevated PI3KC2β expression in 2/4 cases when compared to a normal Type II pneumocyte cell line (PN) (Figure 1B, left panel). TaqMan analysis of *PIK3C2B* expression confirmed that mRNA levels are predictive of protein expression (Figure 1B, right panel).

In neuroblastoma (NB) cell lines, Western blot analysis revealed broad expression of PI3KC2β (Figure 2A). Upon TaqMan analysis *PIK3C2B* was found to be over-expressed at the mRNA level when compared to normal human adrenal gland (5/8 samples with >2-fold expression) (Figure 2B). In primary tumours from children under the age of one year PI3KC2β showed increased expression (Figure 2C). This distribution was mirrored in data obtained from TaqMan analysis, where increased mRNA expression was found in the same patient subgroup (3/19 samples with >2-fold expression) (Figure 2D).

In glioblastoma multiforme (GBM) cell lines and *ex vivo* cultures PI3KC2β was found to be over-expressed in a subset of samples when compared to normal human brain or cerebellum (Figure 2E).

In medulloblastoma (MB) cell lines, heterogeneous PI3KC2β expression was observed (Figure 2F). Microarry analysis of a panel of primary medulloblastoma samples also showed that PI3KC2β was over-expressed in 16/60 samples compared to normal human cerebellum (Supplemental Figure 1).

Together, these data revealed subgroups of tumours and cell lines displaying PI3KC2β over-expression, in which the class II PI3K may play a role in regulating proliferation, survival or migration.

Inhibition of cell proliferation by pharmacological PI3KC2β inhibitors or siRNA targeting PI3KC2β

To gain insight into the contribution of the class II PI3K isoform PI3KC2β in cell proliferation, two different isoform-specific pharmacological inhibitors (PI701 and PI702) were used. The specificity of these inhibitors was verified by *in vitro* PI3K assays using purified recombinant preparation of various PI3K isoforms. The IC_{50} values for specific inhibition of the enzymatic activity of PI3KC2β were 182 nM for PI701 and 652 nM for PI702 while values above 10μM were observed for the other PI3K isoforms (Table I). Both inhibitors also failed to inhibit the activity of mTOR and DNA-PK, with IC_{50} values above 100 μM (data not shown). A screen against a panel of 72 protein kinases *in vitro* confirmed that the two compounds are selective for PI3KC2β. Indeed, they only showed some inhibitory activity, at a concentration of 10 μM *in vitro*, against anaplastic lymphoma kinase (ALK), c-Raf, p38-regulated/activated kinase (PRAK) and tyrosine protein kinase receptor B (TrkB)* (*PI702 only) (data not shown).

The anti-proliferative activity of both inhibitors was investigated in a panel of 28 cell lines and primary cultures from AML, GBM, MB, NB and SCLC. As controls, non-leukemic bone marrow cells (N), two immortalised B cells lines (FIN COS, 41b MI) and an immortalised Type II pneumocyte cell line (PN) were included. Both inhibitors of PI3KC2β inhibited cell proliferation in a dose-dependent manner with IC_{50} values below 10μM (PI701: 16/30 cases; PI702: 9/25 cases) (Figure 3 and Table II). The lowest IC_{50} values were observed in SCLC cell lines, AML blasts and D341 MB cells (Table II). In contrast, the IC_{50} values observed in control cells (FIN COS, 41b MI, N, PN) were above 10 μM. In several cases, cell lines with high PI3KC2β expression were more sensitive to PI701 and PI702, as was the case for AML (U937, NB4), MB (D341), GBM (T98G) and SCLC (H-209, H-510) (Figure 1 and 2, Table II).

Investigation of the effect of PI701 on activation of downstream signalling mediators in AML cell lines showed a dose-dependent decrease in the phosphorylation of key signalling molecules (Figure 4). These include Akt, c-Jun N-terminal kinases (JNK) and ribosomal S6 protein.

Together, these data show that inhibition of PI3KC2β with selective pharmacological inhibitors impairs proliferation in a subset of human cancer cells with IC_{50} values compatible with inhibition of the enzyme. This is in line with the finding that the phosphorylation of key signalling molecules is decreased upon treatment with PI701 in AML.

To validate the results obtained with pharmacological inhibitors, small interfering RNA (siRNA) targeting PI3KC2β was tested in a AML cell line. The specific down-regulation of PI3KC2β expression by siRNA was verified by Western blot analysis (Figure 5A). Decreased expression of PI3KC2β in U937 resulted in a 40% reduction of cell proliferation and was accompanied by increased caspase-3 activity (Figure 5B). The effects of the PI3KC2β siRNA on cell proliferation were less marked in THP1 cells, which displayed lower expression of the enzyme (data not shown).

Targeting PI3KC2β enhances the sensitivity of AML and GBM cells to chemotherapy

We next investigated whether inhibition of PI3KC2β by pharmacological inhibitors could modulate the sensitivity of AML and GBM cells to chemotherapeutic agents. In AML cell lines, combination treatment with PI701 led to markedly increased sensitivity to etoposide (Figure 6A). This effect was also observed in GBM cell lines and *ex vivo* cultures where PI701 combined with doxorubicin had a stronger effect on cell viability than the chemotherapeutic agent alone (Figure 6B and 6C). Moreover, the combination treatment enhanced the induction of apoptosis as assessed by increased caspase-3 activation and poly (ADP-ribose) polymerase (PARP) cleavage (Figure 6D and 6E).

In summary, these results highlight the importance of PI3KC2β in regulating the viability and chemoresistance of various tumour cells.

PI3KC2β contributes to cell migration in transformed epithelial cells

Previous reports have described a role for PI3KC2β in the migration of cancer cells (Katso et al., 2006; Maffucci et al., 2005). To investigate the specific contribution of PI3KC2β in the migration of tumourigenic vs. non-tumourigenic cells, murine epithelial cells sequentially transformed by oncogenic H-Ras and TGF-β1 were used (Maschler et al., 2005). To analyse

the contribution of PI3KC2β to the migratory capacity of cancer cells, murine epithelial cells were treated with PI701. Western blot analysis revealed increased PI3KC2β expression in the FibRas cells, which have previously been shown to display enhanced migration and invasion, as compared to Eph4 and EpRas cells (Maschler et al., 2005) (Figure 7A). Treatment with PI701 impaired migration of FibRas cells but had no significant effect on the migration of Eph4 and EpRas cells (Figure 7B). At the concentrations used, PI701 did not significantly inhibit cell proliferation (data not shown). The finding that pharmacological inhibition of PI3KC2β impaired the migratory capacity of highly invasive cells emphasises the importance of PI3KC2β in regulating cancer cell migration.

DISCUSSION

To date, the class I_A isoform p110α is the only validated target of the PI3K family in the context of human cancer. Other class I_A isoforms have been associated with a role in various malignancies, including p110β in colon cancer, and p110δ in AML and breast cancer. Concerning the class II PI3Ks, PI3KC2α has been shown to play a role in survival of HeLa cells and increased expression of PI3KC2β was reported in a subset of tumours and cell lines from AML, GBM and SCLC (Arcaro et al., 2002; Knobbe & Reifenberger, 2003; Qian et al., 2002). Furthermore, PI3KC2β has been shown to play a role in the migration of A-431, HeLa and ovarian cancer cells, and to contribute to SCLC cell growth and Akt activation in response to growth factors such as SCF (Arcaro et al., 2002; Katso et al., 2006; Maffucci et al., 2005). In the present study, we have extended these studies on PI3KC2β in AML, brain tumours and neuroendocrine tumours.

Here we report that PI3KC2β is overexpressed in a variety of human cancer cell lines and primary cultures compared to control tissue. The growing interest in small molecule inhibitors has led to the development of a plethora of pharmacological PI3K inhibitors whose activity in human cancer remains to be validated. In the present study, the effect of two specific inhibitors of PI3KC2β (PI701, PI702) on cancer cell responses was investigated. Upon treatment with either compound a dose-dependent inhibition of cell proliferation was observed in various human tumour cells while non-tumourigenic cells such as immortalised B cells or type II pneumocytes remained largely unaffected. Moreover, a correlation between protein expression levels and sensitivity to the inhibitors was observed in a number of cell lines. In AML, siRNA against PI3KC2β also led to decreased proliferation accompanied by an increase in apoptosis. The finding that the activation status of important signalling molecules including Akt, JNK and S6K was reduced upon treatment with PI701 is in line with the inhibitory effect of this compound on cell proliferation.

Interestingly, cotreatment of cells with PI701 led to an increased sensitivity to chemotherapeutic agents such etoposide and doxorubicin. This supports previous reports showing that the inhibition of important survival pathways, including PI3K/Akt, can enhance the response to cytotoxic reagents (Abdul-Ghani et al., 2006; Kumar et al., 2005). In the present study we have demonstrated for the first time that PI3KC2β inhibitors can sensitise human cancer cells to chemotherapeutic agents such as etoposide and doxorubicin.

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Besides its role in regulating cell proliferation, in invasive murine breast cancer cell lines PI3KC2β was also found to be involved in cell migration. Enhanced expression of PI3KC2β was detected in the most motile cells (FibRas) as compared to non-invasive cells (EpH4). Treatment of these cells with increasing doses of PI701 strongly reduced the migratory capacity in wound healing assays. This is an interesting observation considering that malignant cells often acquire the ability to migrate leading to the propagation of metastases throughout the body.

In summary, the present study illustrates that PI3KC2β plays a crucial role in regulating various cellular responses in a broad spectrum of human cancer cells. Since the pharmacological inhibitor PI701 not only reduced basal cell proliferation but also had an effect on chemosensitivity and cellular migration, PI3KC2β could prove to be an attractive target for cancer treatment in the future.

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FIGURE LEGENDS

Figure 1 PI3KC2β expression in AML and SCLC cells. Western blot analysis of PI3KC2β expression in (**A**) normal bone marrow cells (N), primary blasts (1-7), AML cell lines and immortalized B cells (FIN COS, 41b MI) and (**B**) type II pneumocytes (PN) and SCLC cell lines (left panel). Relative mRNA expression levels of *PIK3C2B* in (**A**) human AML cell lines and (**B**) SCLC cell lines (right panel). Nontransformed type II human pneumocytes were used as a control.

Figure 2 PI3KC2β expression in tumours of the central nervous system. In (**A-B**) neuroblastoma cell lines and (**C-D**) primary tumour samples, PI3KC2β expression was analysed both by Western blot analysis (A, C) as well as quantitative RT-PCR (B, D). PI3KC2β protein expression in (**E**) glioblastoma cell lines and *ex vivo* cultures and (**F**) medulloblastoma cell lines by Western blot analysis. For neuroblastoma samples, human adrenal tissue (AG) was used as a control. For glioblastoma and medulloblastoma samples, normal human cerebellum served as a control.

Figure 3 Titration curve of PI701 in AML and SCLC. (**A**) AML cell lines (U937, triangles; HL-60, circles) and patient blasts (FAB M1, asterisks) and (**B**) SCLC cell lines (H209, triangles; H510, circles) were treated with increasing concentration of the inhibitor for 72 hours. For (**A**) AML cell lines and blast cells, immortalised B cells (diamonds and squares) served as a control. For (**B**) SCLC, nontransformed type II pneumocytes (squares) were used as a control.

Figure 4 Effect of PI701 on the phosphorylation of downstream signalling molecules. AML cell lines (U937 and THP1) were treated with increasing concentrations of PI701 overnight. Pathway activation was visualised by monitoring the phosphorylation status of Akt (P-AktSer^{473}), JNK (P-JNK$^{Thr183/Tyr185}$) and S6 protein (P-S6 protein$^{Ser235/236}$).

Figure 5 Inhibition of AML cell proliferation by small interfering RNA (siRNA) targeting PI3KC2β. (**A**) U937 cells transfected with control siRNA or siRNA targeting PI3KC2β were analyzed by western blotting for protein expression. (**B**) Cell proliferation of U937 cells

transfected with siRNA targeting PI3KC2β was analysed by MTS assay (left panel) and caspase-3 activity was assessed in parallel. **P<0.01 by analysis of variance test.

Figure 6 PI701 sensitises AML and glioblastoma cells to chemotherapeutical agents. (**A**) Titration curve of U937 (left panel) or THP1 (right panel) cells incubated with increasing concentrations of etoposide in the absence (black circles) or presence (white circles) of PI701 (1μM). (**B**) Titration curve of T98G (left panel) or U251 (right panel) cells incubated with increasing concentrations of doxorubicin in the absence (black circles) or presence (white circles) of PI701 (1μM). (**C**) Titration curve of two glioblastoma *ex vivo* cultures incubated with increasing concentrations of doxorubicin in the absence (black circles) or presence (white circles) of PI701 (1μM). A representative experiment (out of three) performed with eight repetitions is shown for A-C. *P<0.05 or **P<0.01 by analysis of variance test. (**D**) T98G cells were treated with PI701 or doxorubicin alone or in combination and caspase-3 cleavage was evaluated by means of a Western blot. (**E**) U937 cells were treated with increasing concentration of etoposide either alone or in combination with PI701 and PARP cleavage was visualised by means of a Western blot. The band corresponding to cleaved PARP (85 kDa) is shown.

Figure 7 Role of PI3KC2β expression in mammary epithelial cell lines. (**A**) Western blot analysis of PI3KC2β expression in EpH4, EpRas and FibRas cells. (**B**) Effect of PI701 on migration of mammary epithelial cell lines. Cells were treated with increasing concentrations of PI701 and the distance migrated was measured after 8 hours.

Supplemental Figure 1 Microarray analysis of PI3KC2β expression in a panel of primary medulloblastoma samples and cell lines. Gene expression profiles were obtained by using the Affymetrix HG-U133 Plus 2.0 array.

REFERENCES

Abdul-Ghani, R., Serra, V., Gyorffy, B., Jurchott, K., Solf, A., Dietel, M. & Schafer, R. (2006). The PI3K inhibitor LY294002 blocks drug export from resistant colon carcinoma cells overexpressing MRP1. *Oncogene*, **25,** 1743-52.

Alessi, D.R., Deak, M., Casamayor, A., Caudwell, F.B., Morrice, N., Norman, D.G., Gaffney, P., Reese, C.B., MacDougall, C.N., Harbison, D., Ashworth, A. & Bownes, M. (1997). 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. *Curr Biol*, **7,** 776-89.

Arcaro, A., Khanzada, U.K., Vanhaesebroeck, B., Tetley, T.D., Waterfield, M.D. & Seckl, M.J. (2002). Two distinct phosphoinositide 3-kinases mediate polypeptide growth factor-stimulated PKB activation. *Embo J*, **21,** 5097-108.

Arcaro, A., Volinia, S., Zvelebil, M.J., Stein, R., Watton, S.J., Layton, M.J., Gout, I., Ahmadi, K., Downward, J. & Waterfield, M.D. (1998). Human phosphoinositide 3-kinase C2beta, the role of calcium and the C2 domain in enzyme activity. *J Biol Chem*, **273,** 33082-90.

Arcaro, A., Zvelebil, M.J., Wallasch, C., Ullrich, A., Waterfield, M.D. & Domin, J. (2000). Class II phosphoinositide 3-kinases are downstream targets of activated polypeptide growth factor receptors. *Mol Cell Biol*, **20,** 3817-30.

Broderick, D.K., Di, C., Parrett, T.J., Samuels, Y.R., Cummins, J.M., McLendon, R.E., Fults, D.W., Velculescu, V.E., Bigner, D.D. & Yan, H. (2004). Mutations of PIK3CA in anaplastic oligodendrogliomas, high-grade astrocytomas, and medulloblastomas. *Cancer Res*, **64,** 5048-50.

Brown, R.A., Domin, J., Arcaro, A., Waterfield, M.D. & Shepherd, P.R. (1999). Insulin activates the alpha isoform of class II phosphoinositide 3-kinase. *J Biol Chem*, **274,** 14529-32.

Cantley, L.C. & Neel, B.G. (1999). New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc Natl Acad Sci U S A*, **96,** 4240-5.

Chantry, D., Vojtek, A., Kashishian, A., Holtzman, D.A., Wood, C., Gray, P.W., Cooper, J.A. & Hoekstra, M.F. (1997). p110delta, a novel phosphatidylinositol 3-kinase catalytic subunit that associates with p85 and is expressed predominantly in leukocytes. *J Biol Chem*, **272,** 19236-41.

Doepfner, K.T., Spertini, O. & Arcaro, A. (2007). Autocrine insulin-like growth factor-I signaling promotes growth and survival of human acute myeloid leukemia cells via the phosphoinositide 3-kinase/Akt pathway. *Leukemia*, **21,** 1921-30.

Domin, J., Harper, L., Aubyn, D., Wheeler, M., Florey, O., Haskard, D., Yuan, M. & Zicha, D. (2005). The class II phosphoinositide 3-kinase PI3K-C2beta regulates cell migration by a PtdIns3P dependent mechanism. *J Cell Physiol*, **205,** 452-62.

Domin, J., Pages, F., Volinia, S., Rittenhouse, S.E., Zvelebil, M.J., Stein, R.C. & Waterfield, M.D. (1997). Cloning of a human phosphoinositide 3-kinase with a C2 domain that displays reduced sensitivity to the inhibitor wortmannin. *Biochem J*, **326 (Pt 1),** 139-47.

Guerreiro, A.S., Boller, D., Shalaby, T., Grotzer, M.A. & Arcaro, A. (2006). Protein kinase B modulates the sensitivity of human neuroblastoma cells to insulin-like growth factor receptor inhibition. *Int J Cancer*, **119,** 2527-38.

Harada, K., Truong, A.B., Cai, T. & Khavari, P.A. (2005). The class II phosphoinositide 3-kinase C2beta is not essential for epidermal differentiation. *Mol Cell Biol*, **25,** 11122-30.

Hawkins, P.T., Jackson, T.R. & Stephens, L.R. (1992). Platelet-derived growth factor stimulates synthesis of PtdIns(3,4,5)P3 by activating a PtdIns(4,5)P2 3-OH kinase. *Nature*, **358,** 157-9.

Hayakawa, M., Kawaguchi, K., Kaizawa, H., Koizumi, T., Ohishi, T., Yamano, M., Okada, M., Ohta, M., Tsukamoto, S., Raynaud, F.I., Parker, P., Workman, P. & Waterfield, M.D. (2007). Synthesis and biological evaluation of sulfonylhydrazone-substituted imidazo[1,2-a]pyridines as novel PI3 kinase p110alpha inhibitors. *Bioorg Med Chem*, **15,** 5837-44.

Hiles, I.D., Otsu, M., Volinia, S., Fry, M.J., Gout, I., Dhand, R., Panayotou, G., Ruiz-Larrea, F., Thompson, A., Totty, N.F. & et al. (1992). Phosphatidylinositol 3-kinase: structure and expression of the 110 kd catalytic subunit. *Cell*, **70,** 419-29.

Hu, P., Mondino, A., Skolnik, E.Y. & Schlessinger, J. (1993). Cloning of a novel, ubiquitously expressed human phosphatidylinositol 3-kinase and identification of its binding site on p85. *Mol Cell Biol*, **13,** 7677-88.

Inukai, K., Anai, M., Van Breda, E., Hosaka, T., Katagiri, H., Funaki, M., Fukushima, Y., Ogihara, T., Yazaki, Y., Kikuchi, Oka, Y. & Asano, T. (1996). A novel 55-kDa regulatory subunit for phosphatidylinositol 3-kinase structurally similar to p55PIK Is generated by alternative splicing of the p85alpha gene. *J Biol Chem*, **271,** 5317-20.

Katso, R., Okkenhaug, K., Ahmadi, K., White, S., Timms, J. & Waterfield, M.D. (2001). Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Annu Rev Cell Dev Biol*, **17,** 615-75.

Katso, R.M., Pardo, O.E., Palamidessi, A., Franz, C.M., Marinov, M., De Laurentiis, A., Downward, J., Scita, G., Ridley, A.J., Waterfield, M.D. & Arcaro, A. (2006). Phosphoinositide 3-Kinase C2beta regulates cytoskeletal organization and cell migration via Rac-dependent mechanisms. *Mol Biol Cell*, **17,** 3729-44.

Knobbe, C.B. & Reifenberger, G. (2003). Genetic alterations and aberrant expression of genes related to the phosphatidyl-inositol-3'-kinase/protein kinase B (Akt) signal transduction pathway in glioblastomas. *Brain Pathol*, **13,** 507-18.

Kumar, P., Benedict, R., Urzua, F., Fischbach, C., Mooney, D. & Polverini, P. (2005). Combination treatment significantly enhances the efficacy of antitumor therapy by preferentially targeting angiogenesis. *Lab Invest*, **85,** 756-67.

Le Good, J.A., Ziegler, W.H., Parekh, D.B., Alessi, D.R., Cohen, P. & Parker, P.J. (1998). Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science*, **281,** 2042-5.

MacDougall, L.K., Domin, J. & Waterfield, M.D. (1995). A family of phosphoinositide 3-kinases in Drosophila identifies a new mediator of signal transduction. *Curr Biol*, **5,** 1404-15.

MacDougall, L.K., Gagou, M.E., Leevers, S.J., Hafen, E. & Waterfield, M.D. (2004). Targeted expression of the class II phosphoinositide 3-kinase in Drosophila melanogaster reveals lipid kinase-dependent effects on patterning and interactions with receptor signaling pathways. *Mol Cell Biol*, **24,** 796-808.

Maffucci, T., Cooke, F.T., Foster, F.M., Traer, C.J., Fry, M.J. & Falasca, M. (2005). Class II phosphoinositide 3-kinase defines a novel signaling pathway in cell migration. *J Cell Biol*, **169,** 789-99.

Maschler, S., Wirl, G., Spring, H., Bredow, D.V., Sordat, I., Beug, H. & Reichmann, E. (2005). Tumor cell invasiveness correlates with changes in integrin expression and localization. *Oncogene*, **24,** 2032-41.

Misawa, H., Ohtsubo, M., Copeland, N.G., Gilbert, D.J., Jenkins, N.A. & Yoshimura, A. (1998). Cloning and characterization of a novel class II phosphoinositide 3-kinase containing C2 domain. *Biochem Biophys Res Commun*, **244,** 531-9.

Molz, L., Chen, Y.W., Hirano, M. & Williams, L.T. (1996). Cpk is a novel class of Drosophila PtdIns 3-kinase containing a C2 domain. *J Biol Chem*, **271,** 13892-9.

Ono, F., Nakagawa, T., Saito, S., Owada, Y., Sakagami, H., Goto, K., Suzuki, M., Matsuno, S. & Kondo, H. (1998). A novel class II phosphoinositide 3-kinase predominantly expressed in the liver and its enhanced expression during liver regeneration. *J Biol Chem*, **273,** 7731-6.

Otsu, M., Hiles, I., Gout, I., Fry, M.J., Ruiz-Larrea, F., Panayotou, G., Thompson, A., Dhand, R., Hsuan, J., Totty, N. & et al. (1991). Characterization of two 85 kd proteins that associate with receptor tyrosine kinases, middle-T/pp60c-src complexes, and PI3-kinase. *Cell*, **65,** 91-104.

Pardo, O.E., Arcaro, A., Salerno, G., Tetley, T.D., Valovka, T., Gout, I. & Seckl, M.J. (2001). Novel cross talk between MEK and S6K2 in FGF-2 induced proliferation of SCLC cells. *Oncogene*, **20,** 7658-67.

Pons, S., Asano, T., Glasheen, E., Miralpeix, M., Zhang, Y., Fisher, T.L., Myers, M.G., Jr., Sun, X.J. & White, M.F. (1995). The structure and function of p55PIK reveal a new regulatory subunit for phosphatidylinositol 3-kinase. *Mol Cell Biol*, **15,** 4453-65.

Qian, Z., Fernald, A.A., Godley, L.A., Larson, R.A. & Le Beau, M.M. (2002). Expression profiling of CD34+ hematopoietic stem/ progenitor cells reveals distinct subtypes of therapy-related acute myeloid leukemia. *Proc Natl Acad Sci U S A*, **99,** 14925-30.

Samuels, Y., Wang, Z., Bardelli, A., Silliman, N., Ptak, J., Szabo, S., Yan, H., Gazdar, A., Powell, S.M., Riggins, G.J., Willson, J.K., Markowitz, S., Kinzler, K.W., Vogelstein, B. & Velculescu, V.E. (2004). High frequency of mutations of the PIK3CA gene in human cancers. *Science*, **304,** 554.

Sansal, I. & Sellers, W.R. (2004). The biology and clinical relevance of the PTEN tumor suppressor pathway. *J Clin Oncol*, **22,** 2954-63.

Stephens, L.R., Hughes, K.T. & Irvine, R.F. (1991). Pathway of phosphatidylinositol(3,4,5)-trisphosphate synthesis in activated neutrophils. *Nature*, **351,** 33-9.

Valk, P.J., Verhaak, R.G., Beijen, M.A., Erpelinck, C.A., Barjesteh van Waalwijk van Doorn-Khosrovani, S., Boer, J.M., Beverloo, H.B., Moorhouse, M.J., van der Spek, P.J., Lowenberg, B. & Delwel, R. (2004). Prognostically useful gene-expression profiles in acute myeloid leukemia. *N Engl J Med*, **350,** 1617-28.

Vanhaesebroeck, B. & Alessi, D.R. (2000). The PI3K-PDK1 connection: more than just a road to PKB. *Biochem J*, **346 Pt 3,** 561-76.

Vanhaesebroeck, B., Leevers, S.J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P.C., Woscholski, R., Parker, P.J. & Waterfield, M.D. (2001). Synthesis and function of 3-phosphorylated inositol lipids. *Annu Rev Biochem*, **70,** 535-602.

Vanhaesebroeck, B., Leevers, S.J., Panayotou, G. & Waterfield, M.D. (1997a). Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem Sci*, **22,** 267-72.

Vanhaesebroeck, B. & Waterfield, M.D. (1999). Signaling by distinct classes of phosphoinositide 3-kinases. *Exp Cell Res*, **253,** 239-54.

Vanhaesebroeck, B., Welham, M.J., Kotani, K., Stein, R., Warne, P.H., Zvelebil, M.J., Higashi, K., Volinia, S., Downward, J. & Waterfield, M.D. (1997b). P110delta, a novel phosphoinositide 3-kinase in leukocytes. *Proc Natl Acad Sci U S A*, **94,** 4330-5.

Wang, Y., Yoshioka, K., Azam, M.A., Takuwa, N., Sakurada, S., Kayaba, Y., Sugimoto, N., Inoki, I., Kimura, T., Kuwaki, T. & Takuwa, Y. (2006). Class II phosphoinositide 3-kinase alpha-isoform regulates Rho, myosin phosphatase and contraction in vascular smooth muscle. *Biochem J*, **394,** 581-92.

Weir, B., Zhao, X. & Meyerson, M. (2004). Somatic alterations in the human cancer genome. *Cancer Cell*, **6,** 433-8.

Table I IC_{50} values (μM) against isolated enzymes.

Compound	PI3KC2β	p110α	p110β	p110γ	p110δ
PI701	**0.528**	**> 10**	**> 10**	**> 10**	**> 10**
PI702	**0.632**	**> 10**	**> 10**	**> 10**	**> 10**

Table II IC_{50} values (μM) of PI701 and PI702 against a panel of cancer cell lines and primary cultures from NB, GBM, MB, AML, and SCLC.

IC50 Values		
	PI701	**PI702**
Neuroblastoma Cell Lines		
CHP134	7.4	8.0
LAN1	8.3	10.0
SHSY5Y	15.8	6.6
WAC2	5.2	6.0
Glioblastoma Cell Lines and *ex vivo* Cultures		
T98G	6.9	> 20
U251	8.0	> 20
LN319	19.6	14
LN229	6.2	14.1
U87	> 20	10.2
LO	11.6	ND
RE	13.2	ND
SB	18.2	ND
Medulloblastoma Cell Lines		
DAOY	10.1	> 20
D341	4.5	4.0
AML Cell Lines, Patient Blasts and Control Cells		
U937	3.2	5.5
HL-60	9.6	12.4
NB4	5.3	7.8
THP1	8.4	9.5
Kasumi	> 20	ND
KG-1	17.0	8.0
K562	10.4	> 20
FIN COS	> 20	> 20
41b MI	> 20	> 20
FAB M5	2.5	ND
FAB M1	3.6	ND
FAB M0	ND	6.2
N	ND	12.6
SCLC Cell Lines and Control Cells		
H69	4.4	ND
H209	4.3	ND
H510	5.0	ND
SW2	10.2	ND
PN	12.0	ND

LO, RE, SB = glioblastoma *ex vivo* cultures; FIN COS, 41b MI = immortalised B cells; FAB = French-American-British classification; N = non-leukemic bone marrow cells; PN = immortalised Type II pneumocytes; ND = not determined

Figure 1

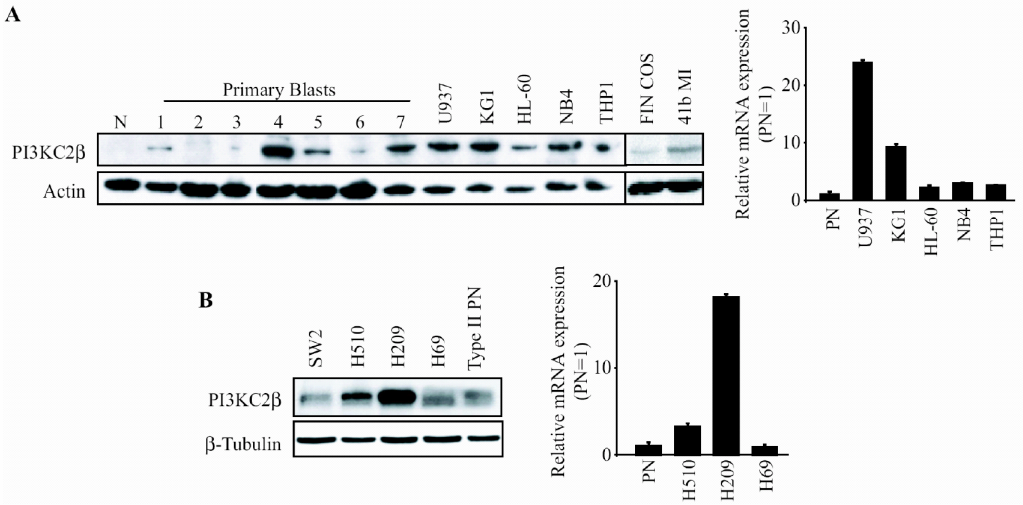
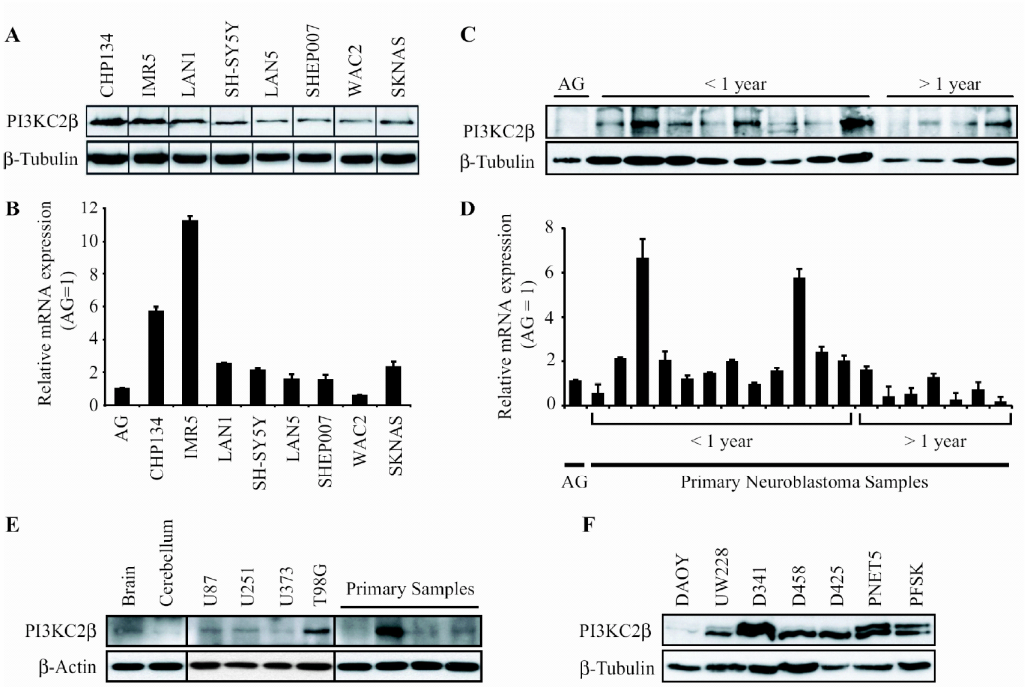
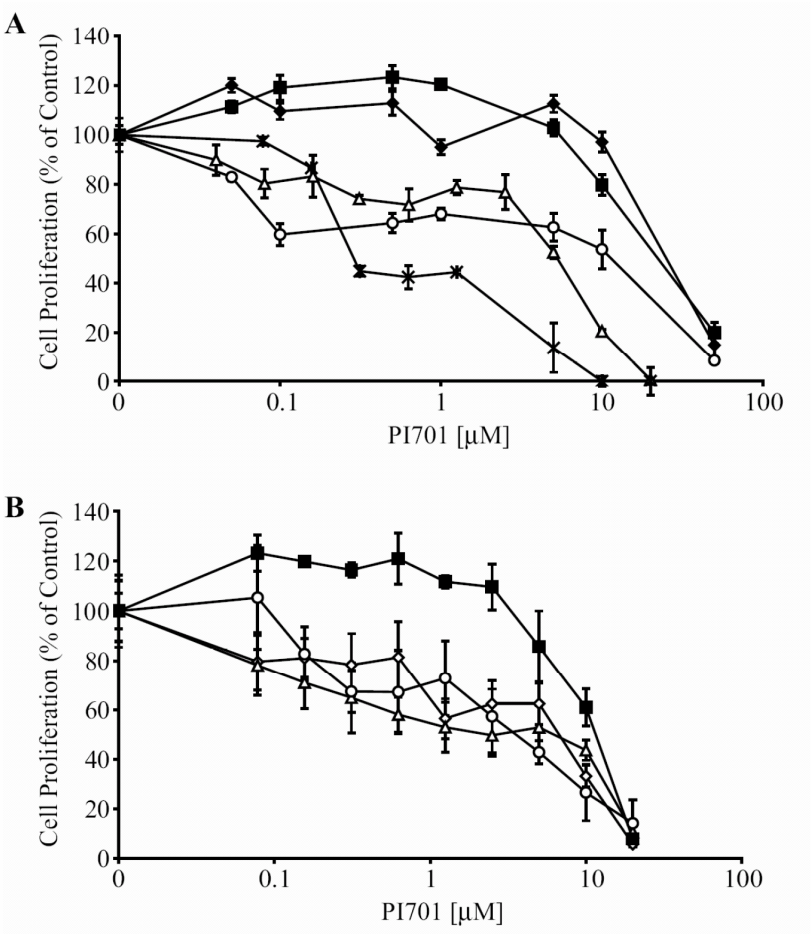

A
Primary Blasts
N
1
2
3
4
5
6
7
U937
KG1
HL-60
NB4
THP1
FIN COS
41b MI
PI3KC2β
Actin
Relative mRNA expression (PN=1)
30
20
10
0
PN
U937
KG1
HL-60
NB4
THP1
B
SW2
H510
H209
H69
Type II PN
PI3KC2β
β-Tubulin
Relative mRNA expression (PN=1)
20
10
0
PN
H510
H209
H69

Figure 2


A
CHP134
IMR5
LAN1
SH-SY5Y
LAN5
SHEP007
WAC2
SKNAS
PI3KC2β
β-Tubulin
C
AG
< 1 year
> 1 year
PI3KC2β
β-Tubulin
B
Relative mRNA expression (AG=1)
12
10
8
6
4
2
0
AG
CHP134
IMR5
LAN1
SH-SY5Y
LAN5
SHEP007
WAC2
SKNAS
D
Relative mRNA expression (AG = 1)
8
6
4
2
0
< 1 year
> 1 year
AG
Primary Neuroblastoma Samples
E
Brain
Cerebellum
U87
U251
U373
T98G
Primary Samples
PI3KC2β
β-Actin
F
DAOY
UW228
D341
D458
D425
PNET5
PFSK
PI3KC2β
β-Tubulin

25

Figure 3


A
Cell Proliferation (% of Control)
140
120
100
80
60
40
20
0
0
0.1
1
10
100
PI701 [µM]
B
Cell Proliferation (% of Control)
140
120
100
80
60
40
20
0
0
0.1
1
10
100
PI701 [µM]

26

Figure 4

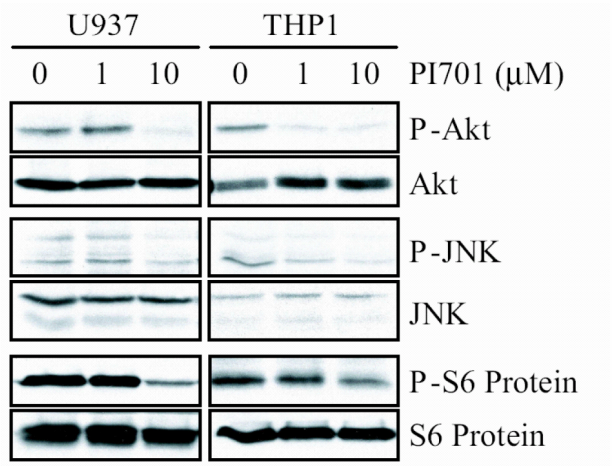

U937
THP1
0 1 10 0 1 10 PI701 (μM)
P-Akt
Akt
P-JNK
JNK
P-S6 Protein
S6 Protein

Figure 5

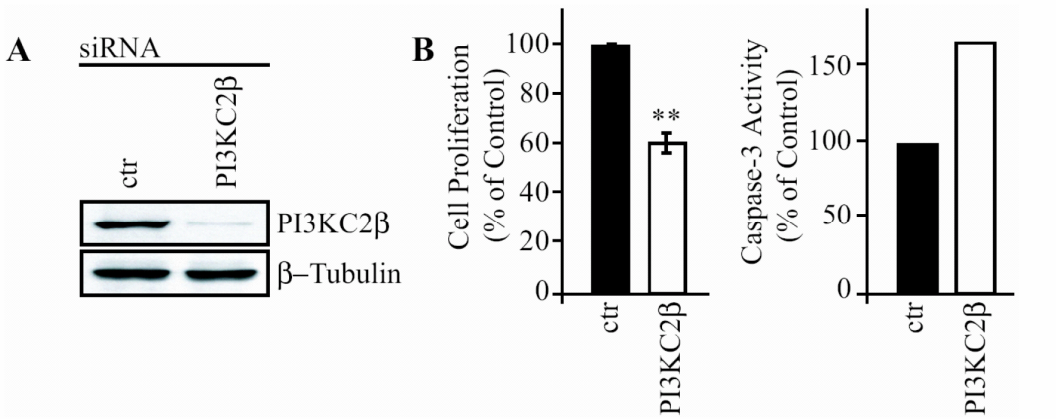

A
siRNA
ctr
PI3KC2β
PI3KC2β
β–Tubulin
B
Cell Proliferation (% of Control)
100
80
60
40
20
0
**
ctr
PI3KC2β
Caspase-3 Activity (% of Control)
150
100
50
0
ctr
PI3KC2β

Figure 6

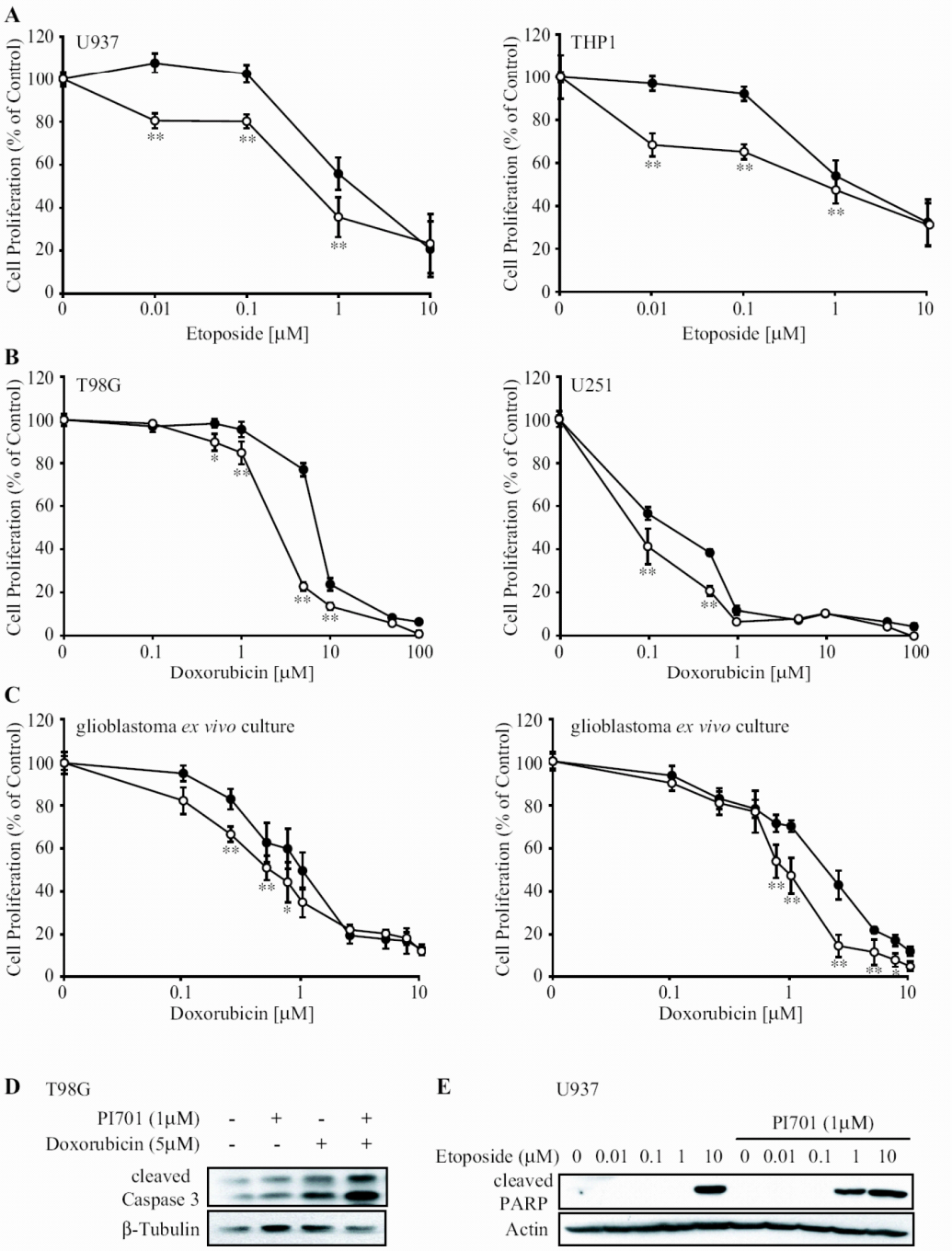
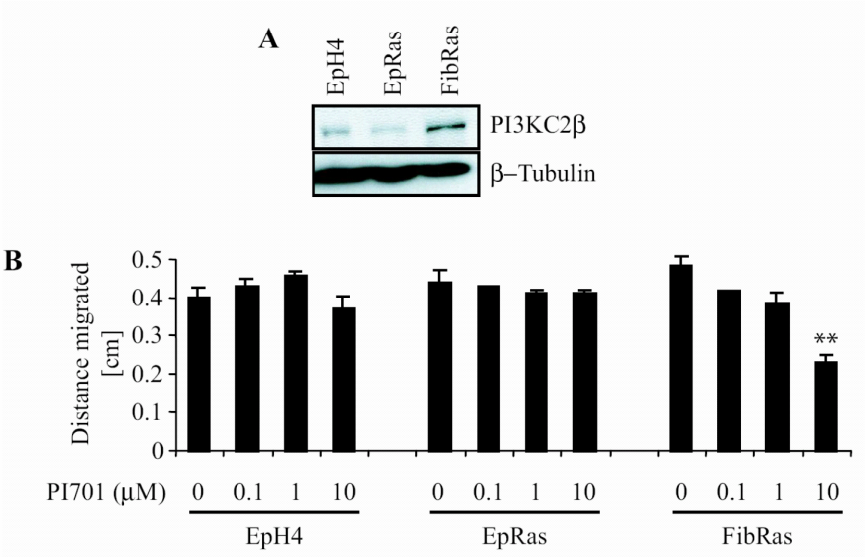
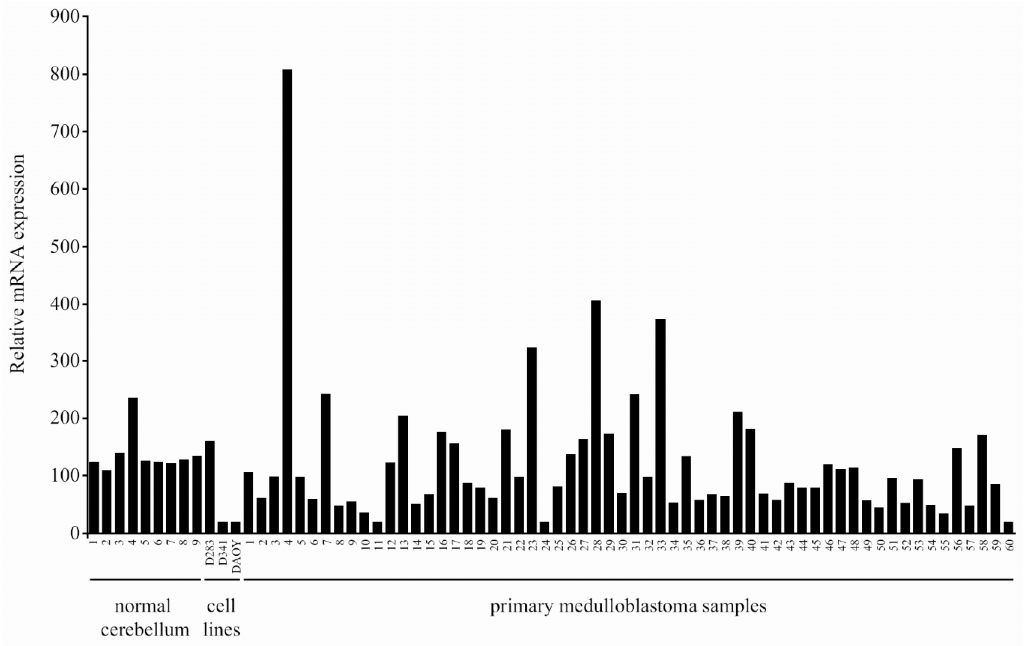

A
U937
THP1
Cell Proliferation (% of Control)
Etoposide [µM]
B
T98G
U251
Doxorubicin [µM]
C
glioblastoma *ex vivo* culture
D
T98G
PI701 (1µM)
Doxorubicin (5µM)
cleaved Caspase 3
β-Tubulin
E
U937
PI701 (1µM)
Etoposide (µM)
cleaved PARP
Actin

Figure 7


A
EpH4
EpRas
FibRas
PI3KC2β
β–Tubulin
B
Distance migrated [cm]
0.5
0.4
0.3
0.2
0.1
0
**
PI701 (μM) 0 0.1 1 10 0 0.1 1 10 0 0.1 1 10
EpH4
EpRas
FibRas

Supplemental Figure 1



3.4. Investigation of the Molecular Determinants of Rapamycin Sensitivity in Acute Myeloid Leukemia Cells (Manuscript in preparation)

1

Investigation of the Molecular Determinants of Rapamycin Sensitivity in Acute Myeloid Leukemia Cells

Running Title: Rapamycin sensitivity in AML

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ABSTRACT

The mammalian target of rapamycin (mTOR) is a major regulator of cell cycle, proliferation and cell growth. In acute myeloid leukemia (AML) cells, protein expression analysis revealed significant differences in the expression levels of mTOR. Therefore, the role of mTOR in AML cell growth and survival was investigated and cellular responses to the mTOR inhibitor rapamycin were compared in cells expressing high levels of mTOR ($mTOR^{high}$ cells) and cells expressing low levels of mTOR ($mTOR^{low}$ cells). Moreover, an RNAi screen was aimed at uncovering kinases which modulate the sensitivity to rapamycin. Growth factor stimulation revealed impaired phosphorylation of the ribosomal S6 protein and 4E-binding protein (4E-BP) in $mTOR^{low}$ cells. Upon transfection of these cells with a construct encoding mTOR phosphorylation of S6 protein was restored. Nevertheless, rapamycin treatment resulted in a comparable growth reduction and induction of apoptosis in $mTOR^{high}$ and $mTOR^{low}$ cells. Down-regulation of mTOR by siRNA, however, reduced cell growth in $mTOR^{high}$ cells only. Together, these data show an important role of mTOR in a subclass of human AML cells and describe potential molecular targets for cancer therapy in combination with the mTOR inhibitor rapamycin.

INTRODUCTION

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that controls fundamental cellular processes including transcription, translation, cell size, cell growth, and cytoskeletal organization. TOR was initially isolated by a genetic screen in yeast as a target of the antifungal and immunosuppressant agent rapamycin, a macrocyclic lactone (Heitman et al., 1991). The high specificity of rapamycin for TOR has helped to elucidate the structure and function of this protein and a high conservation of TOR and its signaling network from yeast to mammals could be described. Whereas in *S. cerevisiae* two genes called *TOR1* and *TOR2* could be found (Heitman et al., 1991; Helliwell et al., 1994), one single mammalian homologue, mTOR (also known as RAFT, FRAP and RAPT) was uncovered (Hay & Sonenberg, 2004). Using chemical crosslinking and immunoaffinity purification, mTOR-associated proteins and two different multi-protein complexes, mTORC1 and mTORC2 could be identified in human. The multi-protein complex mTORC1 contains the core components mTOR, mLST8/GβL (G protein β-subunit-like protein) and raptor (Hara et al., 2002; Inoki & Guan, 2006). The activity of mTORC1 is regulated by the integration of signals such as nutrients, growth factors, energy availability and cellular stressors (e.g. hypoxia, osmotic stress, viral infections) (Corradetti & Guan, 2006; Reiling & Sabatini, 2006), and is potently inhibited by rapamycin in complex with the FK506-binding protein FKBP12. Rapamycin together with FKBP12 potently binds to the FRB (FBP12-rapamycin) domain located N-terminal to the kinase domain of TOR leading to the inhibition of its function (Fingar & Blenis, 2004). The mTORC2 complex consists of mTOR, mLST8/GβL and rictor and is believed to be rapamycin-insensitive (Jacinto et al., 2004). Recent findings, however, suppose that prolonged rapamycin exposure (> 24h) can disrupt mTORC2 assembly and function by sequestering newly synthesized mTOR molecules (Sarbassov et al., 2006).

Abnormal activation of signaling pathways both proximal and distal to mTOR frequently occurs in human cancer making this kinase an attractive target for cancer therapies. Different human tumor suppressor proteins such as the tuberous sclerosis complexes (TSC1 and TSC2) or the phosphatase and tensin homologue (PTEN) are known to tightly regulate mTOR. Loss of these tumor suppressors leads to dysregulation of mTOR signaling and results in uncontrolled cell growth and proliferation driving tumorigenesis (Sabatini, 2006; Tee & Blenis, 2005). The acute myeloid leukemia (AML), a cancer of the hematopoietic system, is characterized by an arrest in the differentiation pathway of myeloid progenitors that results in cells that are unable to differentiate into mature functional cells, in particular red blood cells,

leukocytes, and platelets. The development of AML is thought to occur upon two classes of crucial mutational events (Gilliland & Tallman, 2002). Alteration of receptor tyrosine kinase (RTK) signaling (class I mutations) together with mutations in hematopoietic transcription factors (class II mutations) result in increased survival and/or proliferation advantages and are required for the genesis of this leukemia. A constitutive activation of signaling pathways involving mTOR was shown to commonly occur in AML patients (Kubota et al., 2004; Martelli et al., 2006; Min et al., 2003; Zhao et al., 2004). Inhibition of mTOR in AML cells resulted in significant growth inhibition and cell cycle blockage in the G_0/G_1 phase (Recher et al., 2005). Interestingly, the anti-proliferative effect was most pronounced in immature AML cell lines and significant anti-leukemic responses could be induced in poor-risk AML patients upon rapamycin therapy (Recher et al., 2005).

In the present study, the role and function of mTOR and the molecular determinants of rapamycin sensitivity were investigated in AML cells. Expression levels of mTOR were studied in a panel of AML cell lines and the role of this protein on AML cell growth and survival was analyzed. Cells expressing high levels of mTOR were compared to cells expressing low levels of mTOR. Moreover, screening an RNAi kinome library was aimed at identifying human kinases that sensitize AML cells to rapamycin in order to uncover potential new candidates for cancer therapy in combination with the already widely used mTOR inhibitor.

MATERIALS AND METHODS

Reagents and antibodies

Antibodies and reagents were purchased from the following companies: FRAP/mTOR, Akt, ERK, S6 protein, 4E-binding protein, PARP, caspase-3 (Santa Cruz Biotechnology, SantaCruz, CA, USA); activated Akt/PKB (Ser473), activated S6 protein (Ser235/236), activated ERK (42/44), activated 4E-binding protein (Cell Signalling Technology, Danvers, MA, USA); β -actin (Sigma-Aldrich, St Louis, MO, USA); human si*ARRAY* SMARTpool protein kinase library, siGENOME™ siRNA (Dharmacon, Lafayette, CO, USA); NVP-AEW541 (Novartis Pharma AG, Basel, Switzerland); AG1296, piceatannol (Calbiochem, La Jolla, CA, USA); imatinib (BIAFFIN GmbH & Co KG, Kassel, Germany).

Apoptosis

For detection of apoptosis, cells were incubated for 16-24 hrs in the presence or absence of inhibitors. The cells were lysed and caspase-3 activity was measured using the CaspACE Assay System (Promega). Additionally, samples were analysed by SDS-PAGE and Western blot with anti-caspase-3 or anti-poly(ADP-ribose) polymerase (PARP) antibodies.

Cell culture

Acute myeloid leukemia cell lines were grown in RPMI (Life Technologies/InvitrogenCarlsbad, CA, USA) with 10% (v/v) fetal calf serum (FCS) and penicillin/streptomycin/L-glutamine, and passaged every 3-5 days. For growth factor stimulations cells were incubated overnight in their growth medium with low serum (0.5-1% v/v) or Optimem medium (Life Technologies/Invitrogen) and washed with serum-free medium prior to incubation with growth factors. Heparinized peripheral blood or bone marrow samples were obtained from adult patients with AML Blast cells were isolated as described previously (Doepfner et al., 2007b).

Cell proliferation

Cell lines (5 x 103 cells/well) were seeded in 96-well plates and grown for 72h in serum (10%)-containing medium in the presence or absence of inhibitors. The number of viable cells was analysed by means of an MTS assay using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Data are mean with SD from 8 repetitions.

SDS-PAGE and Western blot analysis

Cellular lysates were prepared as previously described (Guerreiro et al., 2006) separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a hydrophobic polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham Biosciences), and immunoblotted with various antibodies according to the manufacturer's protocol. Chemiluminescence was used for visualization using the enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham Biosciences) according to the manufacturer's protocol.

siRNA screen

AML cells were transfected with small interfering RNA (siRNA) targeting 779 human kinases (human si*ARRAY* SMARTpool protein kinase library) using DharmaFECT transfection reagents (Dharmacon, Lafayette, CO, USA) according to the manufacturer's protocol. 24h post transfection, rapamycin was added and cell proliferation rate was analyzed after 72h by means of a MTS assay.

Transient and stable expression in AML cells

AML cells were transfected with small interfering RNA (siRNA) or small hairpin RNA (shRNA) using the Amaxa Nucleofector system (Amaxa biosystems, Gaithersburg, MD, USA) according to the manufacturer's protocol. After 48h cells were lyzed in cell lysis buffer in order to visualize protein expression by SDS-PAGE and Western blotting. Besides, cells were analyzed for cell proliferation and apoptosis by MTS assay and Caspase-3 measurement 72h after transfection. Alternatively, the cells transfected with the shRNA were resuspended in fresh medium containing puromycin at 2 μg/ml and selected for 3–4 weeks to obtain stable transfectants.

RT–PCR analysis

RNA was isolated using the RNeasy Mini Kit (Qiagen, Santa Cruz, CA, USA) from $1x10^6$ cells. Reverse transcription–polymerase chain reaction (RT–PCR) was performed according to the QIAGEN OneStep RT–PCR protocol. Expression of mTOR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed using the following primers: mTOR-FP, 5'-CTGGGACTCAAATGTGTGCAGTTC-3'; mTOR-RP, 5'-GAACAATAGGGTGAATGA TCCGGG-3'; GAPDH-FP, 5'-AACGTGTCAGTGGTGGACCT-3'; GAPDH-RP, 5'-GGGTGTCGCTGTTGAAGTCA-3'.

RESULTS

AML cells express variable levels of the mTOR protein and show differences in signal transduction

Various reports have described a crucial role for the PI3K/Akt/mTOR cascade in human cancer development and aberrant activation of growth-regulating genes upstream and downstream of mTOR have been described in AML (Kubota et al., 2004; Martelli et al., 2006; Min et al., 2003; Zhao et al., 2004). Nevertheless, activating mutations or over-expression of mTOR have not been described so far, nor has mTOR been described to be targeted by recurrent translocations in AML (Recher et al., 2005). Therefore, protein expression of mTOR was investigated in a panel of AML cells and its role in signal transduction was analyzed.

Interestingly, the mTOR protein was found to be expressed with high variability in a panel of seven leukemic cell lines, as assessed by Western blot analysis (Figure 1A, upper panel). In contrast, a RT-PCR analysis revealed that the mRNA levels of mTOR were essentially comparable between the different AML cell lines (Figure 1A, lower panel). Stimulation of AML cells with stem cell factor (SCF) or insulin-like growth factor I (IGF-I) activated the signal transducer Akt, ribosomal S6 protein and the 4E-binding protein (4E-BP) in cells expressing high levels of mTOR (Figure 1B, left panel). In $mTOR^{low}$ cells, however, growth factor stimulation revealed impaired phosphorylation of the mTOR downstream transducers S6 protein and 4E-BP whereas the phosphorylation of the upstream molecule Akt was detectable (Figure 1B, right panel).

Taken together, these results revealed differences in the expression levels and significance of the mTOR protein regarding cell signaling and cellular responses in a subgroup of AML cell lines.

Rapamycin treatment results in a comparable growth reduction in $mTOR^{high}$ and $mTOR^{low}$ cells

Rapamycin and its analogs are well known inhibitors of mTOR and treatment with rapamycin was shown to inhibit cell proliferation in a large number of cell lines, including AML cells (Recher et al., 2005). Therefore, the effect of rapamycin on cell growth and survival was investigated in cells expressing high levels of mTOR and compared to cells expressing low levels of mTOR.

In a panel of AML cell lines treated with increasing concentrations of rapamycin, no significant differences between the two groups of $mTOR^{high}$ and $mTOR^{low}$ cells could be identified. Both groups contained highly sensitive AML cell lines with IC_{50} between 1-10ng/ml and cell lines that were much less sensitive to rapamycin (IC50 > 100ng/ml) (Figure 2A). In addition to its strong effects on cell proliferation, rapamycin has been shown to induce a cell cycle arrest in G1/G0 and to enhance apoptosis in cancer cells. Analysis of the cell cycle in $mTOR^{high}$ and $mTOR^{low}$ cells did not reveal any striking differences in the amount of dead cells upon treatment with rapamycin. In both groups of AML cell lines, 20ng/ml of the inhibitor lead to an increase in the number of dead cells by around 20% (Figure 2B).

In support of this notion, evaluation of the induction of caspase-3 activity in the two groups of cells after treatment with rapamycin revealed no significant differences suggesting comparable levels of apoptosis induction in $mTOR^{high}$ and $mTOR^{low}$ cells (Figure 2C). However, the G1/G0 cell cycle arrest upon rapamycin treatment was more pronounced in the cells expressing high levels of mTOR compared to the $mTOR^{low}$ cells (Figure 2B).

In summary, rapamycin treatment appears to affect AML cell proliferation and induction of apoptosis independently of the levels of mTOR protein expression.

mTOR is crucial for cell proliferation in $mTOR^{high}$ cells

The striking differences in the expression levels of mTOR in a panel of AML cells lines raised the question of a particular significance of this protein in $mTOR^{high}$ cells. As the use of the mTOR inhibitor rapamycin did not reveal any striking differences between the two groups of cells regarding cellular responses such as cell proliferation or induction of apoptosis, further experiments were aimed at investigating the exact role of the mTOR protein. Therefore, on the one hand, $mTOR^{low}$ cells were transfected with a construct encoding mTOR and on the other hand, mTOR was down-regulated by small interfering RNA (siRNA) targeting mTOR.

Strikingly, upon transfection of $mTOR^{low}$ cells with a construct encoding mTOR (pRK5-mTOR), the phosphorylation of the downstream target S6 protein was restored after 24 hours already (Figure 3A, left panel). Analysis of cell proliferation, however, did not reveal any significant differences in the proliferation rate of the mTOR-transfected cells compared to the cells transfected with a control empty vector (Figure 3A, right panel). Interestingly, the down-regulation of mTOR by siRNA reduced cell proliferation in $mTOR^{high}$ cells but exerted only marginal effects on $mTOR^{low}$ cells (Figure 3B). Whereas the cells expressing only low levels

of mTOR showed no significant difference regarding cell proliferation after down-regulation of the mTOR protein, the cell lines expressing high levels of mTOR significantly reduced their rate of proliferation up to around 50% (Figure 3B).
Together, these results suggest a crucial role of mTOR on cell proliferation in $mTOR^{high}$ cells whereas the subgroup of cells expressing low levels of mTOR is much less dependent on this protein in regard to cell proliferation.

A siRNA screen uncovers kinases which sensitize AML cells to rapamycin

The results obtained above by testing rapamycin in different AML cell lines revealed an overall anti-proliferative and apoptosis inducing effect of this agent, independently of the expression level of the mTOR protein. Therefore, a siRNA screen was aimed at uncovering kinases which modulate the sensitivity of rapamycin and to compare the two groups of cells expressing high or low levels of mTOR.
In a first set-up, two batches of $mTOR^{low}$ HL-60 cells were transfected with a siRNA library targeting 779 human protein and lipid kinases and analyzed for cell proliferation in the absence or presence of rapamycin. The cells transfected with targeted siRNA were compared to cells transfected with a control non-targeting siRNA either in the absence or presence of rapamycin. The data obtained was first used to identify siRNAs which predominantly reduced cell proliferation in combination with rapamycin and thus sensitized the cells to rapamycin. Top candidates could be found in the group of receptor tyrosine kinases such as the insulin like-growth factor receptor I (IGF-IR), fms-like tyrosine kinase 3 (FLT3) and the fibroblast growth factor receptor 1 (FGFR1). Furthermore, signal transducers such as SYK, ZAP70 and AKT1-3 were uncovered as well as the already well known oncogenes in leukemia ABL1 and ABL2 (Figure 4A). Whereas in some cases the down-regulation of the kinase already lead to a decrease in cell proliferation (IGF-IR, FLT3, ABL1), other candidates only reduced the proliferation rate only in combination with rapamycin (Figure 4A). To validate the top candidates, HL-60 cells were transfected with shRNA specifically targeting SYK, ZAP70, FLT3, ABL1, ABL2 or AKT1 and were again analyzed for cell proliferation in the absence or presence of rapamycin. Using this approach, down-regulation of the candidate proteins reduced cell proliferation rate between 10% to around 45%. Nevertheless, the addition of rapamycin further decreased the proliferation (Figure 4B). Interestingly, the analysis of the basal expression of SYK revealed over-expression of the protein in a panel of AML cell lines when compared to control immortalized B cells (Figure 4C).

In summary, screening a protein kinase library uncovered human kinases which sensitize the AML cell line HL-60 to rapamycin. The down-regulation of certain RTKs or RTK signal transducers strengthened the anti-proliferative effect of rapamycin making them interesting candidates for targeted therapies in combination with the mTOR inhibitor.

RTK inhibitors or inhibition of SYK/ZAP70 reduce cell proliferation and sensitize AML cells to rapamycin

In order to investigate the potential of the candidate proteins as molecular targets in combination wit rapamycin, HL-60 cells were treated with the pharmacological inhibitors NVP-AEW541, AG1296, imatinib or piceatannol. The effect on signal transduction and cell proliferation rate was investigated in absence or presence of rapamycin. NVP-AEW541 is a specific IGF-IR kinase inhibitor and has already been shown to potently inhibit AML cell growth alone or in combination with chemotherapeutical agents (Doepfner et al., 2007b; Garcia-Echeverria et al., 2004). AG1296 inhibits the activity of the PDGFR, c-Kit, as well as FLT3 (Tse et al., 2002). Imatinib is a well known RTK inhibitor which potently inhibits the BCR-ABL fusion protein in human leukemias as well as the receptors c-Kit and PDGFR (Buchdunger et al., 1996; Fang et al., 2000). Piceatannol has been reported as a SYK/ZAP70-specific kinase inhibitor with anti-tumerogenic potential in different human cancers (Balaian et al., 2003; Wang et al., 1998).

Treatment of HL-60 cells with increasing concentrations of NVP-AEW541, AG1296, imatinib or piceatannol reduced the phosphorylation of the signal transducers Akt and ribosomal S6 protein in a dose-dependent manner (Figure 5A). Therefore, the effect on cellular responses such as the rate of proliferation upon treatment with the inhibitors was tested. HL-60 cells turned out to be highly sensitive to the RTK inhibitor imatinib (IC50 < 1μM), whereas the cells were much less responsive to NVP-AEW541, AG1296 or piceatannol (IC50 ≥ 10μM) (Figure 5B). The addition of rapamycin, however, significantly reduced cell proliferation rate in all cases (Figure 5B). Already low concentrations of the inhibitors strongly reduced cell proliferation in combination with rapamycin (10ng/ml).

Taken together, the pharmacological inhibitors NVP-AEW541, AG1296, imatinib and piceatannol affect signal transduction in HL-60 cells, reduce cell proliferation and sensitize the cancer cells to rapamycin.

NVP-AEW541, AG1296, imatinib and piceatannol induce apoptosis in AML cells

In a next step, the role of NVP-AEW541, AG1296, imatinib and piceatannol on the induction of apoptosis was addressed. HL-60 cells were treated with increasing concentrations of the inhibitors and the effect on apoptotic proteins such as PARP and caspase-3 was investigated. Single treatment with NVP-AEW541, imatinib and piceatannol lead to cleavage of PARP and caspase-3 suggesting an induction of apoptosis at concentrations between 1 to 10μM (Figure 6A). AG1296, on the other hand, did not show any effect on the cleavage of PARP or caspase-3 when used as a single agent (Figure 6A). Again, also the potential use of the inhibitors in combination with rapamycin was investigated. HL-60 cells treated with rapamycin in addition to NVP-AEW541, AG1296, imatinib or piceatannol displayed highly increased caspase-3 activity after 24 hours (Figure 6B). Interestingly, already at low concentrations of the inhibitors (1μM), low concentrations of rapamycin (10ng/ml) significantly increased the activity of caspase-3 as compared to the inhibitor alone (Figure 6B).

Together these data describe the potential of the combinatorial use of the RTK inhibitors NVP-AEW541, AG1296 and imatinib or the SYK/ZAP70 inhibitor piceatannol together with rapamycin as an interesting new anti-proliferative approach in AML.

DISCUSSION

The mammalian target of rapamycin (mTOR) has emerged as an important therapeutic target for cancer (Faivre et al., 2006; Hay, 2005; Rowinsky, 2004). Rapamycin and its derivatives that specifically inhibit mTOR are now being actively evaluated in clinical trials (Faivre et al., 2006; Rowinsky, 2004). However, many cancer cells are resistant to rapamycin and its derivatives. The mechanism of this resistance remains a subject of major therapeutic significance (Easton et al., 2006). It was reported that the inhibition of mTOR by rapamycin triggers the activation of two survival signaling pathways that may contribute to drug resistance. Treatment of human lung cancer cells with rapamycin suppressed the phosphorylation of p70(S6K) and 4E-BP1, indicating an inhibition of mTOR signaling (Sun et al., 2005). Paradoxically, rapamycin also concurrently increased the phosphorylation of both Akt and eIF4E (Sun et al., 2005). The rapamycin-induced phosphorylation of Akt and eIF4E was suppressed by LY294002, suggesting the requirement of PI3K in this process (Sun et al., 2005). The activated Akt and eIF4E appeared to attenuate the growth-inhibitory effects of rapamycin, serving as a negative feedback mechanism. In support of this model, rapamycin combined with LY294002 exhibited enhanced inhibitory effects on the growth and colony formation of cancer cells (Sun et al., 2005). Further work suggested that feedback down-regulation of receptor tyrosine kinase signaling is a frequent event in tumor cells with constitutive mTOR activation (O'Reilly et al., 2006). Reversal of this feedback loop by rapamycin may attenuate its therapeutic effects, whereas combination therapy that ablates mTOR function and prevents Akt activation may have improved antitumor activity (O'Reilly et al., 2006). A recent report showed that rapamycin mediates Akt activation through an IGF-IR-dependent mechanism (Wan et al., 2007). Thus, it was concluded that combinatorial targeting of mTOR and the IGF-IR may be a promising strategy to enhance mTOR-targeted anticancer therapy (Wan et al., 2007).

In the present study, we have systematically investigated the mechanisms underlying the sensitivity or resistance of AML cells to rapamycin. The expression levels of mTOR or the activation status of the mTOR/S6K pathway did not appear to make a major contribution to the sensitivity of AML cell lines to rapamycin. Indeed, AML cells with low mTOR expression levels and low mTOR/S6K pathway activation status were not significantly different from cells with high mTOR expression levels in terms of rapamycin sensitivity. In contrast siRNA-mediated down-regulation of mTOR only significantly affected cell growth in AML cells with high mTOR expression levels.

The use of a siRNA screen to identify modulators of rapamycin sensitivity in AML cells uncovered several potential drug targets for combinatorial approaches. In support of previous reports in other cancer cells, targeting Akt or the IGF-IR by siRNA resulted in a sensitization of AML cells to rapamycin (O'Reilly et al., 2006; Sun et al., 2005; Wan et al., 2007). In addition the siRNA screen uncovered several other RTKs as potential modulators of rapamycin sensitivity of AML cells. These RTKs included the FGFR and FLT3, which have previously been shown to be involved in AML cell responses (Doepfner et al., 2007a). Using pharmacological inhibitors of these RTKs, it was possible to sensitize AML cells to rapamycin, confirming the results obtained with siRNA. Since the ABL kinases were identified in the siRNA screen for modulators of rapamycin sensitivity in AML, the combinatorial use of Gleevec, which inhibits ABL with rapamycin may represent a promising drug combination in AML. The combinatorial use of rapamycin derivatives with RTKs has been reported in other systems, such as in glioblastoma in the case of EGFR inhibitors (Wang et al., 2006). Other non-receptor tyrosine kinases of the SYK/ZAP70 family of kinases were also identified in the screen, which led to the validation of SYK/ZAP70 inhibitors (piceatannol) with rapamycin. It is of interest to note here that SYK has been previously shown to be involved in the activation of the mTOR pathway in lymphoma (Leseux et al., 2006), which was supported by the finding that piceatannol potently impaired activation of the S6K pathway in AML cells.

In summary, the present study described the potential of using RNAi screens to identify novel drug targets for combinatorial use with rapamycin and identified several promising drug combination in AML, which warrant further investigations.

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FIGURE LEGENDS

Figure 1 Acute myeloid leukemia (AML) cell lines show variable expression levels of the mammalian target of rapamycin (mTOR) and differences in the response to growth factors. (**A**) Seven AML cell lines (U937, HL-60, NB4, THP1, K562, Kasumi, KG-1) were analyzed by western blotting (upper panel) and RT-PCR (lower panel) for mTOR. Actin and GAPDH was used as a loading control. (**B**) AML cells expressing high or low levels of mTOR ($mTOR^{high}$, NB4; $mTOR^{low}$, U937) were stimulated with stem cell factor (SCF) and insulin-like growth factor I (IGF-I). Pathway activation was monitored by the phosphorylation status of Akt, S6Protein and the 4E-binding protein (4E-BP).

Figure 2 Inhibition of AML cell proliferation and induction of apoptosis by the mTOR inhibitor rapamycin. (**A**) Cell proliferation rate was analyzed by means of an MTS assay in AML cell lines incubated with increasing concentration of rapamycin. (**B**) AML cells with low or high levels of mTOR ($mTOR^{low}$, U937, HL-60; $mTOR^{high}$, THP1) were incubated with increasing concentrations of rapamycin and DNA fragmentation was analyzed by detection of cells with fractional (Sub-G1) DNA content using propidium iodide (PI) staining and FACS analysis. (**C**) AML cells were incubated with increasing concentrations of NVP-AEW541 and analyzed for caspase-3 activity.

Figure 3 Transfection of AML cells with a construct encoding mTOR or small interfering RNA (siRNA) targeting mTOR. (**A**) AML cells expressing low levels of mTOR were transfected with a construct encoding mTOR (pRK5-mTOR) and analysed for phosphorylation of the downstream target S6Protein by Western blot (left) or cell proliferation by means of an MTS assay (right). (**B**) AML cells with low or high levels of mTOR ($mTOR^{low}$, HL-60, U937; $mTOR^{high}$, THP1, NB4) were transfected with siRNA targeting mTOR. Cell proliferation rate was analyzed by MTS assay (left) and the downregulation of the protein was validated by Western blot (right). **$p<0.01$ by analysis of variance test.

Figure 4 A siRNA screen to uncover kinases which sensitize AML cells to rapamycin. (**A**) HL-60 cells were transfected with a protein kinase library targeting 779 human kinases and cell proliferation rate was analyzed in the absence or presence of rapamycin. Top candidates which reduced cell proliferation in addition to rapamycin are listed in a table. Numbers are

percentage of cells transfected with a non-targeting siRNA in the absence or presence of rapamycin. (**B**) HL-60 cells were transfected with shRNA targeting some of the top candidates from the siRNA screen or a control non-targeting shRNA. Cell proliferation rate was measured in the absence or presence of rapamycin by means of a MTS assay after 72h (upper panel). Downregulation of the protein was validated by Western blot after 48h (lower panel). (**C**) Syk protein expression analyzed by Western blot in a panel of AML cell lines. Immortalized B cells (FIN COS, 41b MI) were used as control cells.

Figure 5 Pharmacological inhibitors targeting candidates of the siRNA screen were used to analyze the effect on pathway activation and proliferation rate in the absence or presence of rapamycin. (**A**) HL-60 cells were treated with increasing concentration of NVP-AEW541, AG1296, imatinib or piceatannol and the phosphorylation of the signal transducer Akt and ribosomal S6 protein was analyzed by Western blot after 24h. (**B**) HL-60 cells were incubated with increasing concentrations of NVP-AEW541, AG1296, imatinib or piceatannol in the absence (black signs) or presence (white signs) of rapamycin and cell proliferation rate was measured by means of a MTS assay after 72h. Alternatively, the number of viable cells was counted after staining with Trypan blue after 72h. *$p<0.05$ or **$p<0.01$ by analysis of variance test.

Figure 6 Pharmacological inhibitors targeting candidates of the siRNA screen were used to analyze the effect on apoptosis. (**A**) HL-60 cells were treated with increasing concentration of NVP-AEW541, AG1296, imatinib or piceatannol and the cleavage of PARP or caspase-3 was analyzed by Western blot after 24h. (**B**) HL-60 cells were incubated with increasing concentrations of NVP-AEW541, AG1296, imatinib or piceatannol in the absence or presence of rapamycin and analyzed for caspase-3 activity after 24h. *$p<0.05$ or **$p<0.01$ by analysis of variance test.

REFERENCES

Balaian, L., Zhong, R.K. & Ball, E.D. (2003). The inhibitory effect of anti-CD33 monoclonal antibodies on AML cell growth correlates with Syk and/or ZAP-70 expression. *Exp Hematol*, **31,** 363-71.

Buchdunger, E., Zimmermann, J., Mett, H., Meyer, T., Muller, M., Druker, B.J. & Lydon, N.B. (1996). Inhibition of the Abl protein-tyrosine kinase in vitro and in vivo by a 2-phenylaminopyrimidine derivative. *Cancer Res*, **56,** 100-4.

Corradetti, M.N. & Guan, K.L. (2006). Upstream of the mammalian target of rapamycin: do all roads pass through mTOR? *Oncogene*, **25,** 6347-60.

Doepfner, K.T., Boller, D. & Arcaro, A. (2007a). Targeting receptor tyrosine kinase signaling in acute myeloid leukemia. *Crit Rev Oncol Hematol*, **63,** 215-30.

Doepfner, K.T., Spertini, O. & Arcaro, A. (2007b). Autocrine insulin-like growth factor-I signaling promotes growth and survival of human acute myeloid leukemia cells via the phosphoinositide 3-kinase/Akt pathway. *Leukemia*, **21,** 1921-30.

Easton, J.B., Kurmasheva, R.T. & Houghton, P.J. (2006). IRS-1: auditing the effectiveness of mTOR inhibitors. *Cancer Cell*, **9,** 153-5.

Faivre, S., Kroemer, G. & Raymond, E. (2006). Current development of mTOR inhibitors as anticancer agents. *Nat Rev Drug Discov*, **5,** 671-88.

Fang, G., Kim, C.N., Perkins, C.L., Ramadevi, N., Winton, E., Wittmann, S. & Bhalla, K.N. (2000). CGP57148B (STI-571) induces differentiation and apoptosis and sensitizes Bcr-Abl-positive human leukemia cells to apoptosis due to antileukemic drugs. *Blood*, **96,** 2246-53.

Fingar, D.C. & Blenis, J. (2004). Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. *Oncogene*, **23,** 3151-71.

Garcia-Echeverria, C., Pearson, M.A., Marti, A., Meyer, T., Mestan, J., Zimmermann, J., Gao, J., Brueggen, J., Capraro, H.G., Cozens, R., Evans, D.B., Fabbro, D., Furet, P., Porta, D.G., Liebetanz, J., Martiny-Baron, G., Ruetz, S. & Hofmann, F. (2004). In vivo antitumor activity of NVP-AEW541-A novel, potent, and selective inhibitor of the IGF-IR kinase. *Cancer Cell*, **5,** 231-9.

Gilliland, D.G. & Tallman, M.S. (2002). Focus on acute leukemias. *Cancer Cell*, **1,** 417-20.

Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N., Hidayat, S., Tokunaga, C., Avruch, J. & Yonezawa, K. (2002). Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell*, **110,** 177-89.

Hay, N. (2005). The Akt-mTOR tango and its relevance to cancer. *Cancer Cell*, **8,** 179-83.

Hay, N. & Sonenberg, N. (2004). Upstream and downstream of mTOR. *Genes Dev*, **18,** 1926-45.

Heitman, J., Movva, N.R. & Hall, M.N. (1991). Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science*, **253,** 905-9.

Helliwell, S.B., Wagner, P., Kunz, J., Deuter-Reinhard, M., Henriquez, R. & Hall, M.N. (1994). TOR1 and TOR2 are structurally and functionally similar but not identical phosphatidylinositol kinase homologues in yeast. *Mol Biol Cell*, **5,** 105-18.

Inoki, K. & Guan, K.L. (2006). Complexity of the TOR signaling network. *Trends Cell Biol*, **16,** 206-12.

Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Ruegg, M.A., Hall, A. & Hall, M.N. (2004). Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat Cell Biol*, **6,** 1122-8.

Kubota, Y., Ohnishi, H., Kitanaka, A., Ishida, T. & Tanaka, T. (2004). Constitutive activation of PI3K is involved in the spontaneous proliferation of primary acute myeloid leukemia cells: direct evidence of PI3K activation. *Leukemia*, **18,** 1438-40.

Leseux, L., Hamdi, S.M., Al Saati, T., Capilla, F., Recher, C., Laurent, G. & Bezombes, C. (2006). Syk-dependent mTOR activation in follicular lymphoma cells. *Blood*, **108,** 4156-62.

Martelli, A.M., Nyakern, M., Tabellini, G., Bortul, R., Tazzari, P.L., Evangelisti, C. & Cocco, L. (2006). Phosphoinositide 3-kinase/Akt signaling pathway and its therapeutical implications for human acute myeloid leukemia. *Leukemia*, **20,** 911-28.

Min, Y.H., Eom, J.I., Cheong, J.W., Maeng, H.O., Kim, J.Y., Jeung, H.K., Lee, S.T., Lee, M.H., Hahn, J.S. & Ko, Y.W. (2003). Constitutive phosphorylation of Akt/PKB protein in acute myeloid leukemia: its significance as a prognostic variable. *Leukemia*, **17,** 995-7.

O'Reilly, K.E., Rojo, F., She, Q.B., Solit, D., Mills, G.B., Smith, D., Lane, H., Hofmann, F., Hicklin, D.J., Ludwig, D.L., Baselga, J. & Rosen, N. (2006). mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res*, **66,** 1500-8.

Recher, C., Beyne-Rauzy, O., Demur, C., Chicanne, G., Dos Santos, C., Mas, V.M., Benzaquen, D., Laurent, G., Huguet, F. & Payrastre, B. (2005). Antileukemic activity of rapamycin in acute myeloid leukemia. *Blood*, **105,** 2527-34.

Reiling, J.H. & Sabatini, D.M. (2006). Stress and mTORture signaling. *Oncogene*, **25,** 6373-83.

Rowinsky, E.K. (2004). Targeting the molecular target of rapamycin (mTOR). *Curr Opin Oncol*, **16,** 564-75.

Sabatini, D.M. (2006). mTOR and cancer: insights into a complex relationship. *Nat Rev Cancer*, **6,** 729-34.

Sarbassov, D.D., Ali, S.M., Sengupta, S., Sheen, J.H., Hsu, P.P., Bagley, A.F., Markhard, A.L. & Sabatini, D.M. (2006). Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell*, **22,** 159-68.

Sun, S.Y., Rosenberg, L.M., Wang, X., Zhou, Z., Yue, P., Fu, H. & Khuri, F.R. (2005). Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. *Cancer Res*, **65,** 7052-8.

Tee, A.R. & Blenis, J. (2005). mTOR, translational control and human disease. *Semin Cell Dev Biol*, **16,** 29-37.

Tse, K.F., Allebach, J., Levis, M., Smith, B.D., Bohmer, F.D. & Small, D. (2002). Inhibition of the transforming activity of FLT3 internal tandem duplication mutants from AML patients by a tyrosine kinase inhibitor. *Leukemia*, **16,** 2027-36.

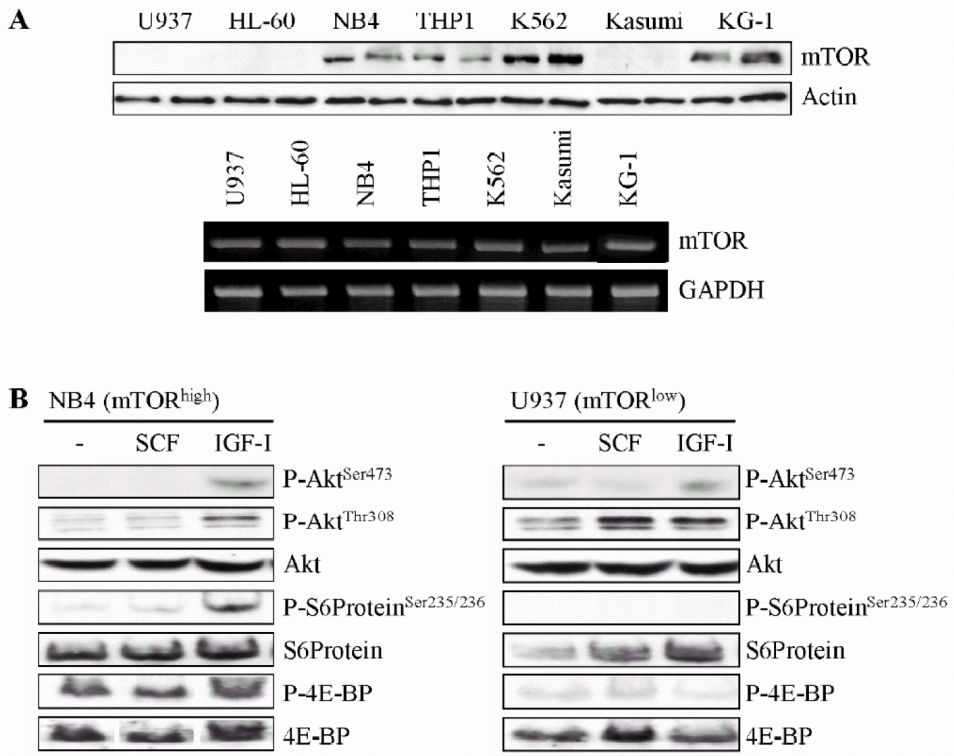
Wan, X., Harkavy, B., Shen, N., Grohar, P. & Helman, L.J. (2007). Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism. *Oncogene*, **26,** 1932-40.

Wang, B.H., Lu, Z.X. & Polya, G.M. (1998). Inhibition of eukaryote serine/threonine-specific protein kinases by piceatannol. *Planta Med*, **64,** 195-9.

Wang, M.Y., Lu, K.V., Zhu, S., Dia, E.Q., Vivanco, I., Shackleford, G.M., Cavenee, W.K., Mellinghoff, I.K., Cloughesy, T.F., Sawyers, C.L. & Mischel, P.S. (2006). Mammalian target of rapamycin inhibition promotes response to epidermal growth factor receptor kinase inhibitors in PTEN-deficient and PTEN-intact glioblastoma cells. *Cancer Res*, **66,** 7864-9.

Zhao, S., Konopleva, M., Cabreira-Hansen, M., Xie, Z., Hu, W., Milella, M., Estrov, Z., Mills, G.B. & Andreeff, M. (2004). Inhibition of phosphatidylinositol 3-kinase dephosphorylates BAD and promotes apoptosis in myeloid leukemias. *Leukemia*, **18,** 267-75.

Figure 1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K562
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KG-1
mTOR
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K562
Kasumi
KG-1
mTOR
GAPDH
B
NB4 (mTORhigh)
U937 (mTORlow)
-
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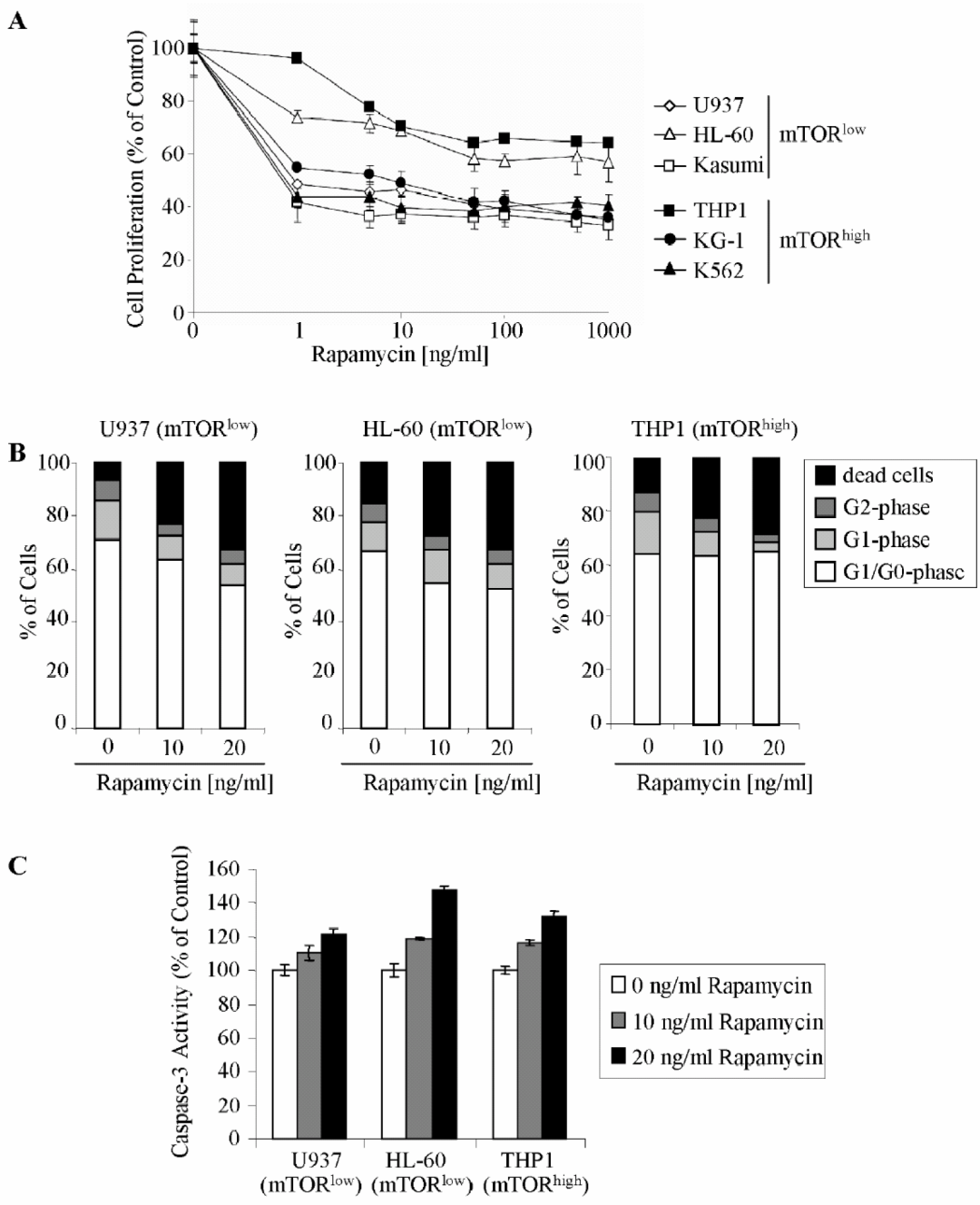
4E-BP

Figure 2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Cell Proliferation (% of Control)
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80
60
40
20
0
0
1
10
100
1000
Rapamycin [ng/ml]
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HL-60
Kasumi
mTOR low
THP1
KG-1
K562
mTOR high
B
U937 (mTOR low)
HL-60 (mTOR low)
THP1 (mTOR high)
% of Cells
0
10
20
Rapamycin [ng/ml]
dead cells
G2-phase
G1-phase
G1/G0-phase
C
Caspase-3 Activity (% of Control)
160
140
120
100
80
60
40
20
0
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THP1 (mTOR high)
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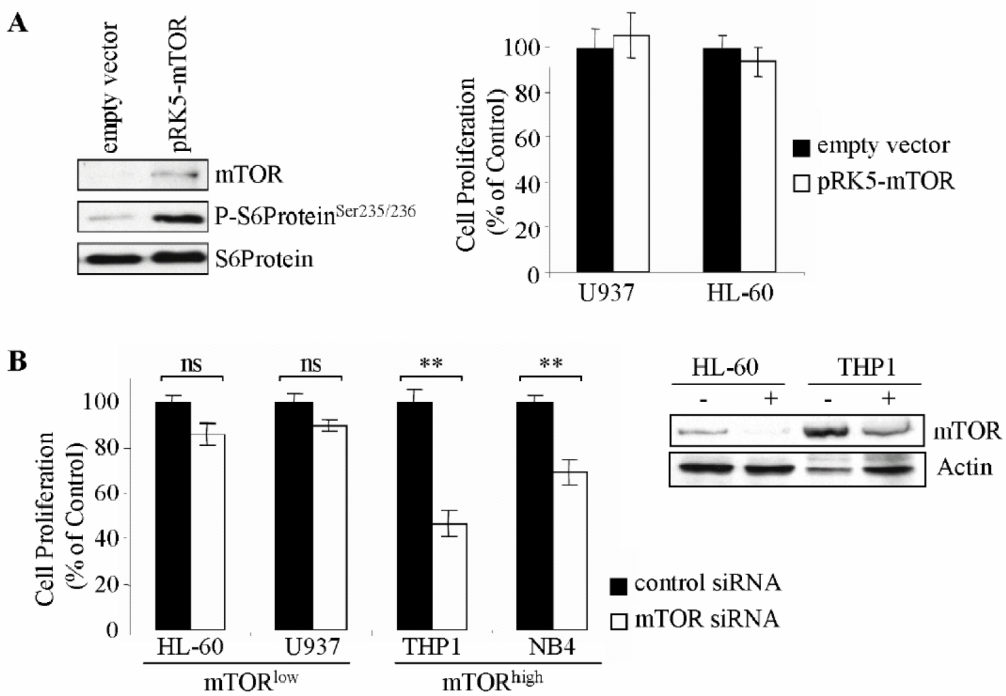
10 ng/ml Rapamycin
20 ng/ml Rapamycin

Figure 3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ns
ns
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Cell Proliferation (% of Control)
HL-60
U937
THP1
NB4
mTOR low
mTOR high
control siRNA
mTOR siRNA
HL-60
THP1
-
+
-
+
mTOR
Actin

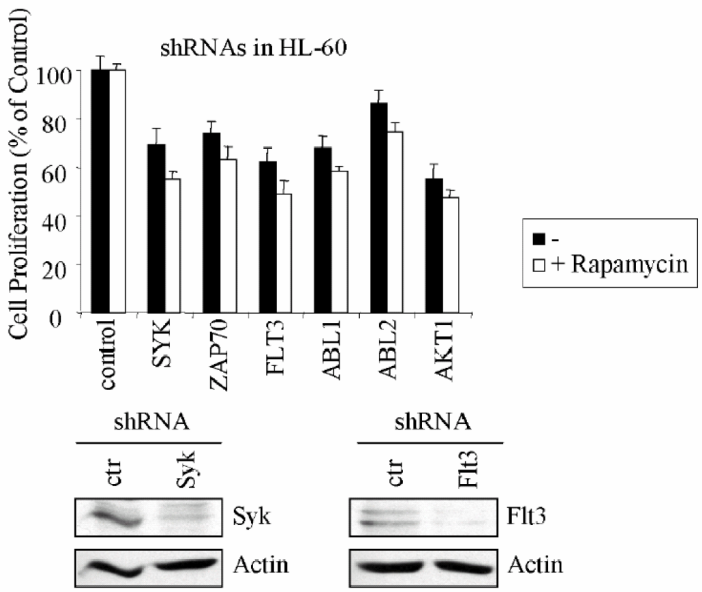
Figure 4

A

siRNA	-	-	+	+	Rapamycin
IGF1R	76.7%	79.9%	67.9%	72.3%	
FLT3	88.6%	91.8%	77.9%	79.7%	
FGFR1	113.4%	120.5%	76.0%	82.5%	
ZAP70	100.6%	90.5%	78.5%	79.4%	
SYK	112.8%	83.0%	79.6%	79.8%	
ABL1	73.9%	93.0%	68.8%	85.0%	
ABL2	94.4%	103.6%	82.7%	81.3%	
AKT1	101.8%	97.5%	89.7%	85.3%	
AKT2	91.7%	99.4%	81.6%	89.4%	
AKT3	101.4%	96.5%	85.2%	90.2%	

100-90%
90-80%
80-70%
70-60%

B



C

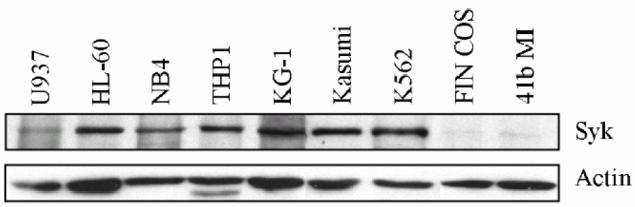
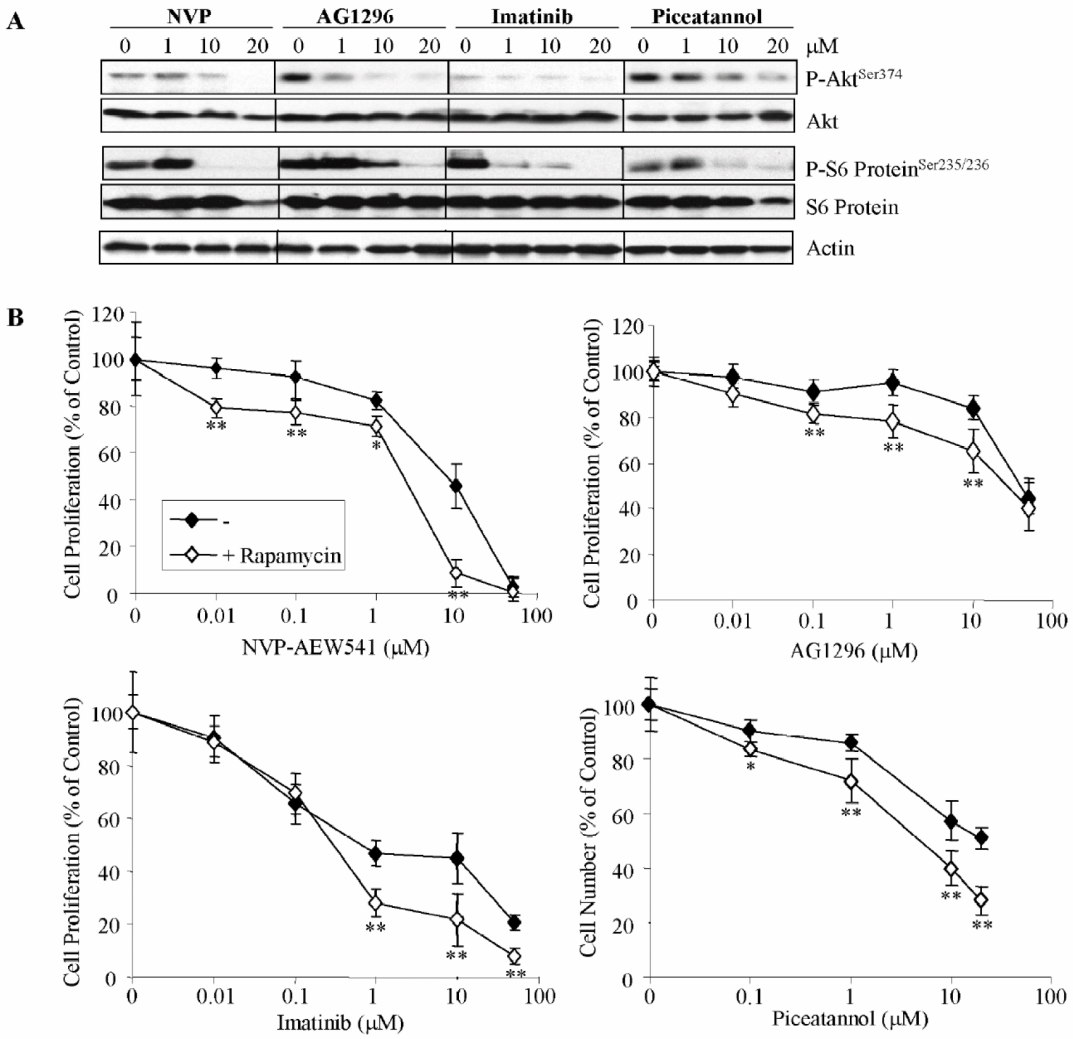


Figure 5


A
NVP
AG1296
Imatinib
Piceatannol
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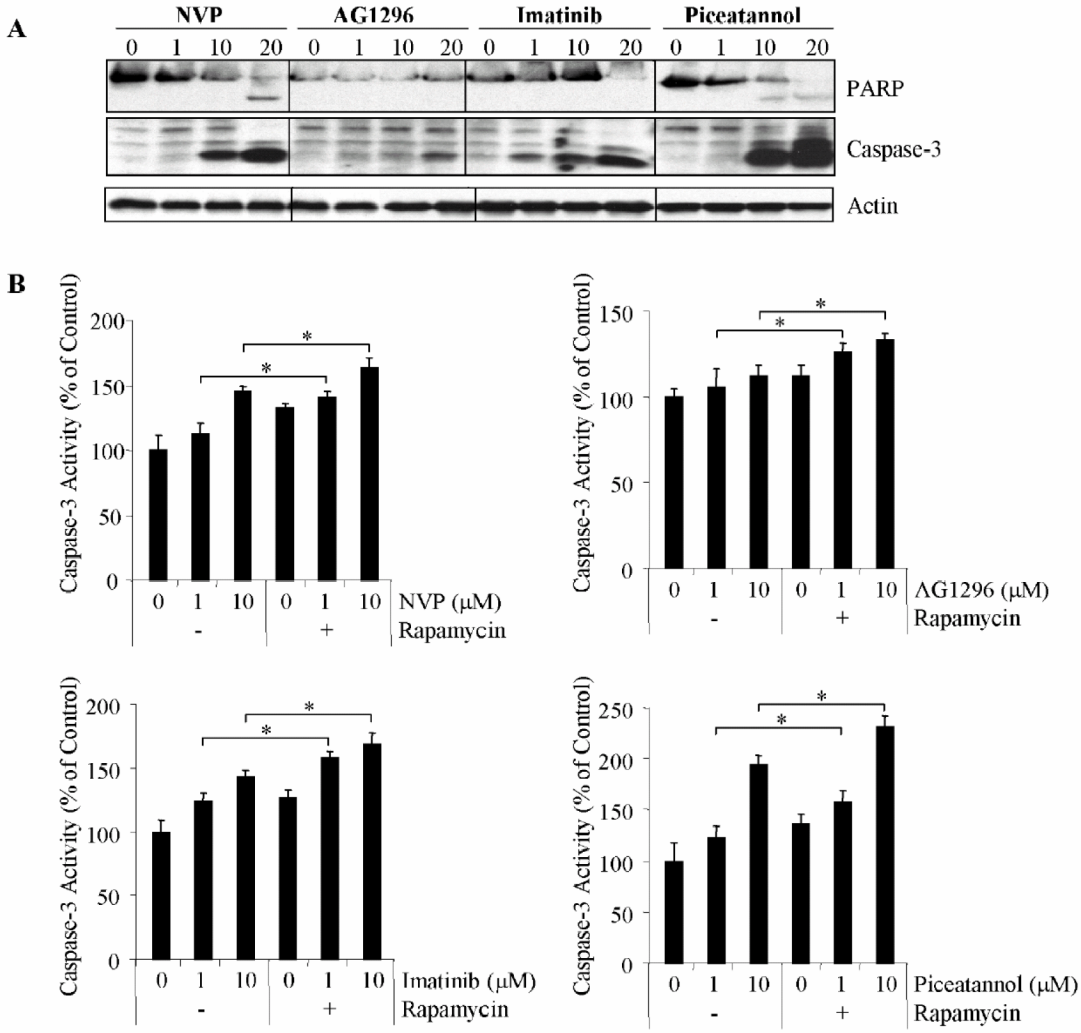
P-Akt Ser374
Akt
P-S6 Protein Ser235/236
S6 Protein
Actin
B
Cell Proliferation (% of Control)
NVP-AEW541 (µM)
AG1296 (µM)
Imatinib (µM)
Cell Number (% of Control)
Piceatannol (µM)
-
+ Rapamycin

Figure 6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NVP
AG1296
Imatinib
Piceatannol
0 1 10 20 0 1 10 20 0 1 10 20 0 1 10 20
PARP
Caspase-3
Actin
B
Caspase-3 Activity (% of Control)
NVP (μM)
AG1296 (μM)
Imatinib (μM)
Piceatannol (μM)
Rapamycin
-
+

3.5. Novel role for insulin as an autocrine growth factor for malignant brain tumour cells

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Novel role for insulin as an autocrine growth factor for malignant brain tumour cells

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AT/RTs (atypical teratoid/rhabdoid tumours) of the CNS (central nervous system) are childhood malignancies associated with poor survival rates due to resistance to conventional treatments such as chemotherapy. We characterized a panel of human AT/RT and MRT (malignant rhabdoid tumour) cell lines for expression of RTKs (receptor tyrosine kinases) and their involvement in tumour growth and survival. When compared with normal brain tissue, AT/RT cell lines overexpressed the IR (insulin receptor) and the IGFIR (insulin-like growth factor-I receptor). Moreover, insulin was secreted by AT/RT cells grown in serum-free medium. Insulin potently activated Akt (also called protein kinase B) in AT/RT cells, as compared with other growth factors, such as epidermal growth factor. Pharmacological inhibitors, neutralizing antibodies, or RNAi (RNA interference) targeting the IR impaired the growth of AT/RT cell lines and induced apoptosis. Inhibitors of the PI3K (phosphoinositide 3-kinase)/Akt pathway also impaired basal and insulin-stimulated AT/RT cell proliferation. Experiments using RNAi and isoform-specific pharmacological inhibitors established a key role for the class I_A PI3K p110α isoform in AT/RT cell growth and insulin signalling. Taken together, our results reveal a novel role for autocrine signalling by insulin and the IR in growth and survival of malignant human CNS tumour cells via the PI3K/Akt pathway.

Key words: Akt, atypical teratoid/rhabdoid tumour (AT/RT), cell proliferation, central nervous system, insulin-like growth factor (IGF), phosphoinositide 3-kinase (PI3K).

INTRODUCTION

The AT/RT (atypical teratoid/rhabdoid tumour) is a highly malignant paediatric tumour of the CNS (central nervous system), which is characterized by unique clinical, biological and histological features [1–4]. Patients with CNS AT/RT respond very poorly to chemotherapy and radiotherapy and thus the prognosis for this particular malignancy is very poor [4–6]. AT/RT is related to other rhabdoid tumours of the kidney [MRT (malignant rhabdoid tumour)] and soft tissues. Most of the rhabdoid tumours harbour inactivating biallelic alterations in the *hSNF5* (Human *SNF5*)/*INI1* gene, a tumour suppressor gene on chromosome 22 [7–9]. hSNF5/INI1 is a component of the ATP-dependent chromatin remodelling SWI/SNF complex [10]. There are multiple sets of mammalian SWI/SNF complexes, with varying subunit compositions, which play important roles in transcriptional regulation, through both activation and repression of gene transcription [10–12]. Mice with a targeted disruption of the *INI1* gene developed tumours at a high frequency and the resulting tumours displayed loss of expression of the hSNF5/INI1 protein [13,14]. Deletion of *hSNF5/INI1* was recently reported to cooperate with p53 loss in oncogenic transformation in murine models [15,16]. One of the mechanisms by which *hSNF5/INI1* exerts its tumour suppressor function was shown to involve repression of cyclin D1 gene expression [17]. Targeting cyclin D1 gene expression was thus suggested to represent a novel therapeutic strategy for AT/RT [17,18].

The insulin/IGF (insulin-like growth factor) family of growth factors are part of an evolutionarily conserved signalling system with a critical role in the growth and development of many tissues as well as the regulation of overall growth and metabolism. This signalling system is characterized by a high complexity and involves multiple proteins including three receptors [IR (insulin receptor), IGFIR (IGF-I receptor) and IGF-II/M-6-PR (mannose 6-phosphate receptor)], three ligands (insulin, IGF-I and IGF-II) and six known types of circulating binding proteins [IGFBP1 (IGF-binding protein 1)–IGFBP6] [19,20]. Both IGF-I and IGF-II bind to the IGFIR, although IGF-I shows a higher affinity than IGF-II [20]. Insulin, the main ligand for the IR, has an IGFIR-binding affinity that is much lower than that of IGF-I [19,20]. The specific receptor for IGF-II, the M-6-PR, differs significantly from the IGFIR, possesses no tyrosine kinase activity and was reported to target IGF-II for lysosomal degradation [21,22]. Signalling by the IGFIR plays a fundamental role in cell growth and malignant transformation and is an important inhibitor of apoptosis [23,24]. The IGFIR is overexpressed in a variety of human tumours including malignant brain tumours [25]. Decreased receptor expression or impaired function was reported to induce a reversal of the transformed phenotype, apoptosis and a decrease in cellular radioresistance and chemoresistance [26]. The IGFIR has thus

Abbreviations used: AT/RT, atypical teratoid/rhabdoid tumour; CNS, central nervous system; Cy3, indocarbocyanine; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EGF, epidermal growth factor; EGFR, EGF receptor; ERK1/2, extracellular-signal-regulated kinase 1/2; FCS, foetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, haemagglutinin; hSNF5, human SNF5; IGF, insulin-like growth factor; IGFBP1, IGF-binding protein 1; IGFIR, IGF-I receptor; IR, insulin receptor; M-6-PR, mannose 6-phosphate receptor; MRT, malignant rhabdoid tumour; mTOR, mammalian target of rapamycin; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; PDX-1, pancreatic duodenal homeobox-1; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; PTEN, phosphatase and tensin homologue deleted on chromosome 10; RNAi, RNA interference; RTKs, receptor tyrosine kinases; RT, reverse transcriptase; SCF, stem cell factor; shRNA, small-hairpin RNA; siRNAs, small interfering RNAs; S6K, S6 kinase; Tos-Lys-CH_2Cl, tosyl-lysylchloromethane.

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been proposed to be a target for the development of novel anti-cancer therapies [26–29]. In medulloblastoma, targeting of the IGFIR with the inhibitor NVP-AEW541 [30] was recently shown to impair cell growth and survival [31]. In AT/RT cells, a recent report has shown that the IGFIR is involved in anti-apoptotic signalling and contributes to chemoresistance [32]. Less is known about the potential involvement of the related IR in human cancer, although its role has been described in the pathogenesis of certain malignancies [33].

A critical intracellular signalling mediator of the IGFIR is the PI3K (phosphoinositide 3-kinase)/Akt [also called PKB (protein kinase B)] pathway [27,34,35]. Indeed, PI3K signalling is implicated in the control of cell proliferation, survival and motility/metastasis downstream of many different growth factor receptors [35,36]. The importance of PI3K signalling in human cancer is highlighted by the fact that mutations in the tumour suppressor gene *PTEN* (phosphatase and tensin homologue deleted on chromosome 10) occur frequently in human tumours [36,37]. PTEN is a phosphatase that antagonizes the action of PI3K by dephosphorylating the D-3 position of polyphosphoinositides [38]. Moreover, previous reports have described activating mutations in the *PIK3CA* gene encoding the catalytic p110α isoform of class I_A PI3K in a variety of human cancers, including, breast, colon and ovarian cancers, as well as medulloblastoma [39,40].

In the present study, we have investigated the expression pattern and biological functions of components of the IR and IGFIR signalling system in human AT/RT and MRT cell lines. Moreover, we have evaluated the potential of targeting the IR or the IGFIR using RNAi (RNA interference), neutralizing antibodies or the inhibitor NVP-AEW541 [30] as an antiproliferative approach in AT/RT cells. Finally, we have investigated whether targeting downstream signalling mediators of the IR could suppress growth and induce apoptosis in AT/RT cell lines. Our findings describe for the first time a role for autocrine signalling by insulin and the IR in growth and survival of AT/RT cells, which involves the PI3K/Akt pathway.

MATERIALS AND METHODS

Reagents and antibodies

Antibodies against Akt/PKB, caspase 3, HA (haemagglutinin) epitope tag, ERK1/2 (extracellular-signal-regulated kinase 1/2), IGFIRβ, INI1, IRβ, lamin B, p110β and p110δ were obtained from Santa Cruz Biotechnology. Antibodies against activated ERK1/2 (Thr^{202}/Tyr^{204}), activated Akt (Ser^{473}), and Thr^{389}-phosphorylated S6K (S6 kinase) were from Cell Signaling Technology. The Akt inhibitor [1L-6-hydroxymethyl-chiro-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate], IGF-I, the IGFIR neutralizing antibody (clone αIR3), LY294002, rapamycin and PD98059 were from Calbiochem. The anti-β-tubulin antibody and insulin were from Sigma–Aldrich. The IR neutralizing antibody (clone 47-9) was from Biosource. The anti-p85α antibody was from Upstate Biotechnology. The antibody against p110α (clone U3A) was a generous gift from Dr A. Klippel (atugen, Berlin, Germany). NVP-AEW541 (Novartis Pharma), YM024, TGX-221 and IC87114 (ICOS Corporation) were dissolved in DMSO at 10 mM and diluted into cell culture medium just prior to use.

Cell lines and cell culture

The human MRT cell lines AS, LP, MON and STM [9] have been described previously. The DAOY medulloblastoma cell line was purchased from the A.T.C.C. (Rockville, MD, U.S.A.). The BT-12 and BT-16 human CNS AT/RT cell lines [32,41] were gifts from Dr Jaclyn Biegel (The Children's Hospital of Philadelphia, PA, U.S.A.). These cell lines have been established from two infants with CNS AT/RT (BT-12 from a 6-week-old female; BT-16 from a 2-year-old male). They have been analysed for *INI1* mutations by Dr Jaclyn Biegel and both contain *INI1* mutations. Human AT/RT cell lines were grown in DMEM (Dulbecco's modified Eagle's medium; Life Technologies/Invitrogen) with 10 % (v/v) FCS (foetal calf serum) and penicillin/streptomycin/L-glutamine, and passaged every 3–5 days by trypsinization. Human MRT cell lines were grown in RPMI 1640 (Life Technologies/Invitrogen) with 10 % FCS and penicillin/streptomycin/L-glutamine. For serum-starving, the cells were incubated for 16 h in DMEM containing 0.5 % FCS. All cell cultures were maintained at 37 °C in a humidified atmosphere with 5 % CO_2.

Cell proliferation and apoptosis

AT/RT cell lines (10^5/ml) were grown in 96-well plates for 3 days in serum (1 or 10 %)-containing medium in the presence or absence of inhibitors. For growth factor stimulations, cells were incubated in medium containing 1 % FCS. Cell proliferation was analysed by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] assay using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega).

For detection of apoptosis, AT/RT cells [1×10^6 cells/well in 6-well plates (35 mm)] were incubated for 24 h in the presence or absence of inhibitors. The cells were then washed in 1× PBS and lysed in 1× gel-loading buffer [50 mM Tris/HCl, pH 6.8, 2 % (w/v) SDS, 100 mM DTT (dithiothreitol), 0.1 % (w/v) Bromophenol Blue and 10 % (w/v) glycerol]. The samples were denatured for 3 min at 100 °C and analysed by SDS/PAGE and Western blot with anti-caspase 3 antibodies.

ELISAs

AT/RT cells were seeded at 5×10^4 cells/well in 6-well plates (35 mm). After 3 h, the cells were washed three times in serum-free medium and incubated in 2 ml of DMEM containing penicillin/streptomycin/L-glutamine. After 5 days, the supernatants were collected, centrifuged to remove cellular contaminants and human insulin was quantified by using the AIA-PACK IRI immunoenzymometric assay (Tosoh Corporation). The background signal caused by the cell culture medium in the absence of cells was subtracted from the measured values.

Immunofluorescence

AT/RT cells were grown on coverslips and serum-starved for 24 h. The cells were then fixed with 4 % (w/v) paraformaldehyde in 1× PBS and permeabilized with methanol for 10 min at −20 °C. The coverslips were blocked with 0.5 % (w/v) non-fat dry milk in 1× PBS for 1 h and incubated with anti-insulin antibody (1:50; Dako) or anti-IRβ (1:100; Santa Cruz Biotechnology) for 1 h. The detection was performed using secondary anti-guinea-pig antibody coupled with FITC, or anti-rabbit antibody coupled with Cy3 (indocarbocyanine). Pictures were taken at ×100 magnification with a Zeiss Axioskop fluorescence microscope.

Immunoprecipitations

AT/RT cells grown to confluence on 10 cm dishes were serum-starved and stimulated with growth factors for 10 min at 37 °C. After washing with ice-cold 1× PBS, the cells were lysed for 20 min on ice in 1 ml of lysis buffer [20 mM Hepes/NaOH,

pH 7.4, 150 mM NaCl, 1 % (w/v) Triton X-100, 2 mM EDTA, 10 mM sodium fluoride, 10 % (w/v) glycerol, 1 mM PMSF, 5 mM benzamidine, 1 mM Tos-Lys-CH_2Cl (tosyl-lysylchloromethane), 10 μM leupeptin, 10 μM pepstatin A, 2 mM Na_3VO_4 (sodium orthovanadate), 10 mM 2-glycerophosphate and 10 mM NaF]. Immunoprecipitation was performed for 2 h at 4 °C with primary antibodies (diluted according to the manufacturer's instructions). Protein G–Sepharose CL-4B (Amersham Biosciences) was then added, and the incubation was continued for 1 h at 4 °C. The immunoprecipitates were washed three times in lysis buffer and resuspended in 1× gel-loading buffer. The samples were denatured for 3 min at 100 °C and analysed by SDS/PAGE and Western blot.

MS analysis

Samples (10 μl) were desalted using Millipore C18 ZipTips. An aliqust of 0.5 μl was applied directly to a prespotted AnchorChip™ target. After 3 min of incubation, 7 μl of washing solution (0.1 % trifluoroacetic acid) was added on the analyte solution and the whole droplet was removed after a few seconds. MALDI–TOF (matrix-assisted laser-desorption ionization–time-of-flight) mass spectra were acquired in linear mode using a Bruker Daltonics Autoflex™ mass spectrometer.

RT (reverse transcriptase)–PCR

Total cellular RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. For each RT–PCR, 2 μg of total RNA was used with the Qiagen One-Step RT–PCR kit. The following primers were used: insulin primer, sense 5′-CGTTCCCCGCACACTAGGTT-3′ and antisense 5′-GCAGCCTTTGTGAACCAACAC-3′; IGF-I primer, sense 5′-GTGCTGCTTTTGTGATTTCTT-3′ and antisense 5′-GTCTTGGGCATGTCGGTGTGG-3′; IGF-II primer, sense 5′-ATGGGGAAGTCGATGCTGGTG-3′ and antisense 5′-ACGGGGTATCTGGGGAAGTTG-3′; PDX-1 (pancreatic duodenal homeobox-1) primer, sense 5′-CTGCGGAGCCGGAGGAGAAC-3′ and antisense 5′-TCTAGAACTACACAGAGAGC-3′; GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primer, sense 5′-GAAGGTGAAGGTCGGAGTC-3′ and antisense 5′-GAAGATGGTGATGGGATTTC-3′. The reaction conditions were as follows: reverse transcription at 50 °C for 30 min, initial PCR activation at 95 °C for 15 min followed by 40 cycles at 95 °C for 15 s, 55 °C for 20 s and 72 °C for 40 s. The PCR products were analysed in 3 % (w/v) agarose gel.

SDS/PAGE and Western-blot analysis

Cellular lysates were prepared with lysis buffer [50 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 % Triton X-100, 1 % (w/v) deoxycholic acid, 0.1 % SDS, 10 % glycerol, 10 μM leupeptin, 10 μM pepstatin A, 1 mM PMSF, 5 mM iodoacetamide, 5 mM benzamidine, 1 mM Tos-Lys-CH_2Cl, 2 mM Na_3VO_4, 10 mM 2-glycerophosphate and 10 mM NaF]. The cells were lysed for 20 min on ice, scraped and lysates were centrifuged at 12000 ***g*** for 20 min at 4 °C. The supernatants were collected and normalized for protein content by using the BCA (bicinchoninic acid) protein assay kit (Pierce). One volume of 2× gel-loading buffer [100 mM Tris/HCl, pH 6.8, 4 % SDS, 200 mM DTT, 0.2 % (w/v) Bromophenol Blue and 20 % (w/v) glycerol] was then added to the samples, followed by denaturation for 3 min at 100 °C and analysed by SDS/PAGE. The gels were transferred on to a hydrophobic PVDF membrane (Hybond-P; Amersham Biosciences) by electrophoresis. The membranes were then blocked in 1× PBS, 5 % (w/v) BSA (phospho-specific antibodies) or 1× PBS/3 % non-fat dry milk (all other antibodies) for 16 h at 4 °C. The membranes were incubated with primary antibodies (diluted according to the manufacturer's protocol) for 16 h at 4 °C. After washing in 1× PBS and 0.1 % (w/v) Tween 20, the immunoblots were incubated with donkey anti-rabbit IgG or sheep anti-mouse IgG secondary antibodies (1:10000 dilution) coupled with horseradish peroxidase (Amersham Biosciences) for 1 h at room temperature. After washing of the immunoblots, chemiluminescence was used for detection, using the ECL® (enhanced chemiluminescence) Western blotting detection reagents (Amersham Biosciences) according to the manufacturer's protocol.

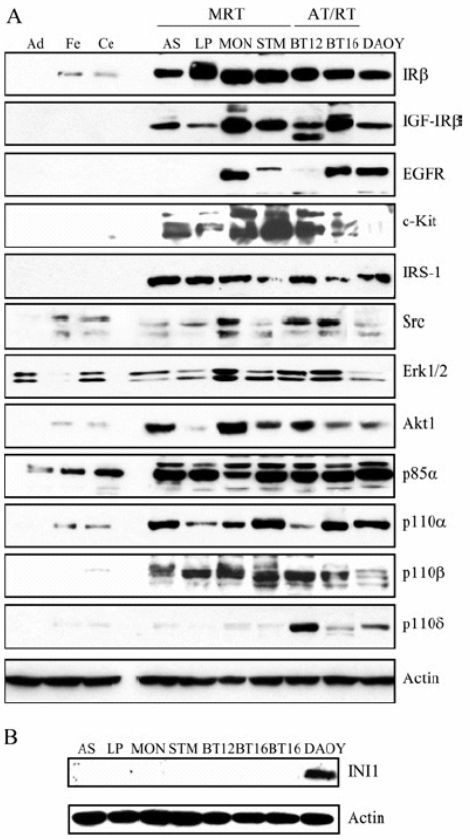

Figure 1 Expression of the IR and its downstream signalling components in MRT and AT/RT cells

(**A**) Equal amounts of lysates from adult brain (Ad), foetal brain (Fe), foetal cerebellum (Ce), or the cell lines indicated were analysed by Western blot for expression of the proteins indicated. The cell lines analysed were AS, LP, MON, STM (MRT), BT-12, BT-16 (AT/RT) and DAOY (medulloblastoma). (**B**) Equal amounts of lysates from the cell lines indicated were analysed by Western blot for expression of INI1.

Transient and stable transfections in AT/RT cells

Human AT/RT cells were transiently transfected with Akt/PKB constructs in pUSE (Upstate Biotechnology), or with PI3K shRNA (small-hairpin RNA) constructs in pRETRO-SUPER [42] by using Lipofectamine™ 2000 (Invitrogen). The transfections

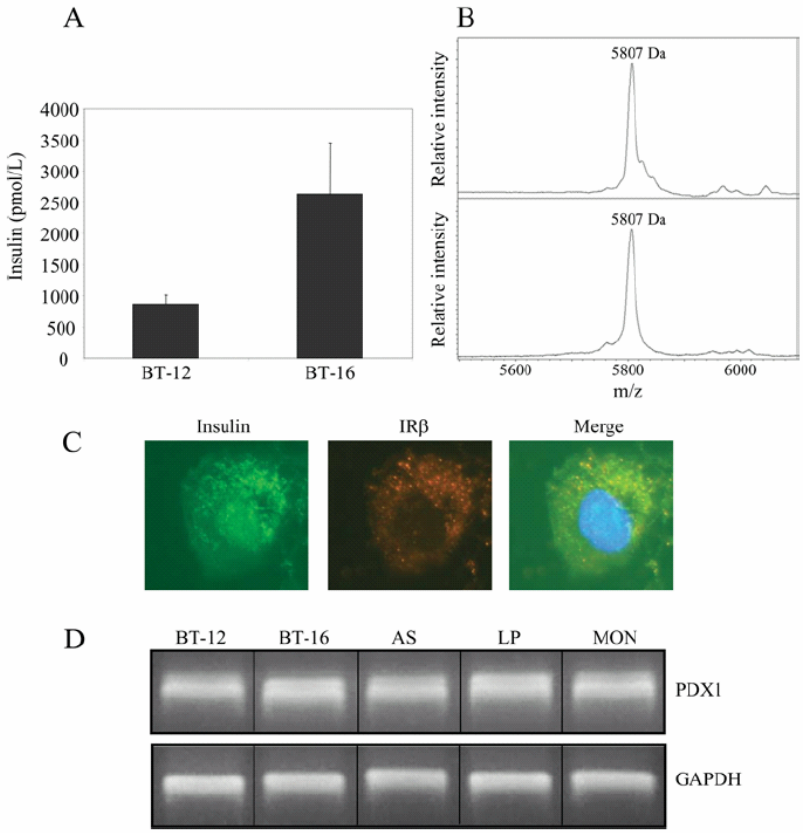

Figure 2 Human AT/RT cells secrete insulin under serum-free conditions

(**A**) Serum-free supernatants from AT/RT cells were analysed after 5 days in culture by ELISA to detect insulin production. Results are the means with S.D. from a representative experiment (out of three) performed in triplicate. (**B**) Serum-free supernatants from BT-16 cells (upper panel) or purified recombinant human insulin (lower panel) were analysed by MS. (**C**) Expression of insulin (left) and the IRβ (centre) in BT-16 cells was investigated by immunofluorescence ($\times$ 100 magnification). The detection was performed using secondary antibodies coupled with FITC (insulin) or Cy3 (IRβ). The right panel shows the merged pictures and the cell nucleus stained with DAPI (4′,6-diamidino-2-phenylindole). (**D**) The expression of PDX-1 was detected in MRT and AT/RT cells using RT–PCR. GAPDH expression was analysed in parallel as a loading control.

were performed in 96-well plates (MTS assays) or 6-well plates (Western-blot analysis), using the amounts of DNA and Lipofectamine™ 2000 recommended by the manufacturer's protocol. Opti-MEM I medium (Invitrogen) was used for the transfection and replaced by growth medium after 24 h.

The siRNAs (small interfering RNAs) targeting the IRβ and IGFIRβ and non-targeting control siRNA were purchased from Dharmacon. The siRNAs were transfected into BT-12 or BT-16 cells using Lipofectamine™ 2000 (Invitrogen). The transfections were performed in 96-well plates (MTS assays) or 6-well plates (Western-blot analysis), using the amounts of siRNA and Lipofectamine™ 2000 recommended by the manufacturer's protocol. Opti-MEM I medium (Invitrogen) was used for the transfection and replaced by growth medium after 24 h. Cell responses were assessed 72 h post-transfection.

For stable expression BT-16 cells (in 10 cm dishes) were transfected with an HA-tagged INI1 construct in pcDNA3 (Invitrogen) or with empty vector using Lipofectamine™ (Invitrogen). The amounts of DNA and Lipofectamine™ used were as recommended by the manufacturer's protocol. After 48 h, the cells were diluted 1:10 in growth medium containing G418 (1.0 mg/ml). A population of resistant cells was expanded and analysed after selection. Western-blot analysis was used to confirm expression of HA-tagged INI1.

RESULTS

Expression of components of the IR and IGFIR signalling pathways in AT/RT cells

We characterized a panel of established human AT/RT cell lines (BT-12 and BT-16) [32,41], MRT lines (AS, LP, MON and STM) [9] and medulloblastoma (DAOY) for expression of RTKs (receptor tyrosine kinases) to study further their involvement in tumour cell proliferation and survival. Lysates from normal adult brain, foetal brain and foetal cerebellum were analysed in parallel as controls. Western-blot analysis revealed that MRT and AT/RT cell lines overexpressed the IR and IGFIR, as compared with the normal brain samples (Figure 1A). Expression of the EGFR [EGF (epidermal growth factor) receptor], ErbB2 and c-Kit were also detected in the panel of cell lines (Figure 1A and results not shown). In contrast, the PDGFR (platelet-derived growth factor receptor) was not detected in the cell lines under study (results not shown). The expression of components of the PI3K/Akt signalling pathway in the panel of MRT and AT/RT cell lines was then

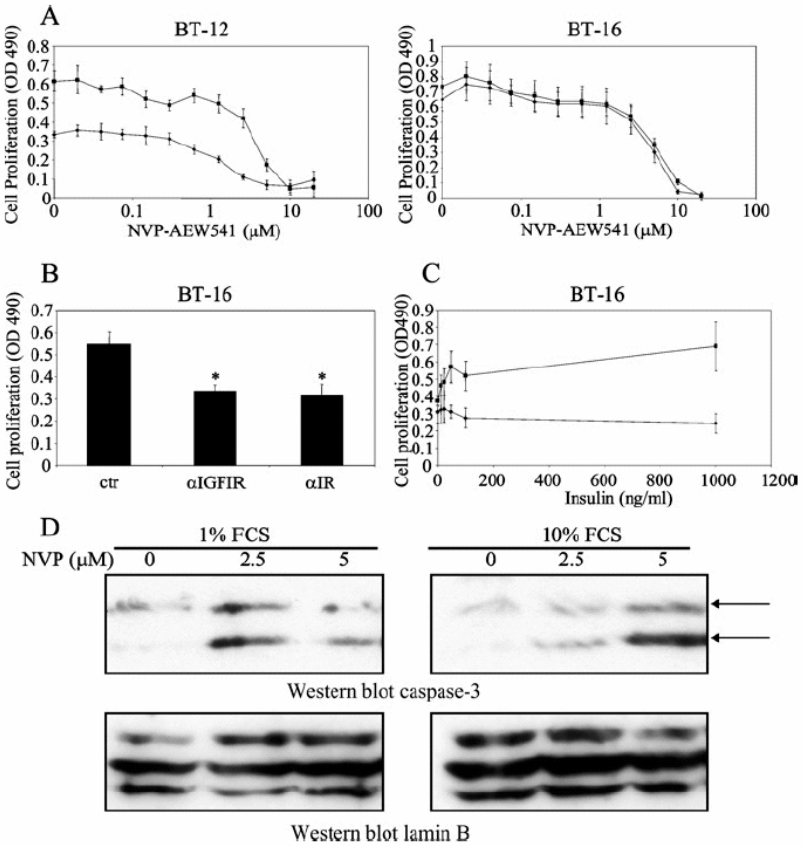

Figure 3 A pharmacological inhibitor or a neutralizing antibody targeting the IR impairs AT/RT cell proliferation

(**A**) AT/RT cells were incubated in the presence of increasing concentrations of the inhibitor NVP-AEW541 in a medium containing 10 % (squares) or 0.5 % (diamonds) FCS. Cell proliferation was determined after 72 h. Results are the means with S.D. from a representative experiment (out of three) performed with eight repetitions. (**B**) BT-16 cells were incubated in serum-free medium in the presence of neutralizing antibodies targeting either the IGFIR or the IR (10 μg/ml) and cell proliferation measured after 72 h. Results are the means with S.D. from three experiments performed in quadruplicate (*, $P < 0.05$ by ANOVA test). (**C**) BT-16 cells were incubated with increasing concentrations of insulin in serum-free medium in the presence of LY294002 (10 μM, diamonds) or vehicle (squares). Cell proliferation was measured after 72 h. Results are the means with S.D. from a representative experiment (out of three) performed with eight repetitions. (**D**) BT-12 cells were incubated for 20 h in the presence of increasing concentrations of the inhibitor NVP-AEW541 and the induction of apoptosis assessed by Western blot for the active fragments of caspase 3 (20 and 17 kDa, arrows). The blots were reprobed for lamin B as a loading control.

investigated. All cell lines expressed the PI3K regulatory subunit p85α and the p110α and p110β catalytic subunits (Figure 1A). In addition, both AT/RT cell lines overexpressed the p110δ isoform (Figure 1A). MRT and AT/RT cell lines displayed comparable levels of IRS-1 (IR substrate-1), mTOR (mammalian target of rapamycin) and ERK1/2 (Figure 1A and results not shown). The AT/RT and MRT cell lines did not express INI1, in contrast with DAOY medulloblastoma cells, as expected (Figure 1B). Indeed, AT/RT and MRT cell lines and tumours harbour inactivating biallelic alterations in the *hSNF5/INI1* gene, leading to loss of expression of the INI1 protein [1,9]. In contrast, these mutations are not found in medulloblastoma [43].

Human AT/RT secrete insulin in an autocrine manner

We next investigated whether AT/RT cells produced growth factors in an autocrine fashion. An ELISA revealed that both BT-12 and BT-16 cell lines secreted insulin into the growth medium, when cultivated under serum-free conditions (Figure 2A). The BT-16 cell line appeared to produce higher amounts of insulin than BT-12 (2628 pmol/l versus 867 pmol/l). IGF-I or IGF-II secretion could not be detected in either BT-12 or BT-16 cells grown in serum-free medium (results not shown). However, an RT–PCR analysis revealed that IGF-I mRNA was expressed in BT-16 cells, while IGF-II expression was detectable in BT-12 cells (see Supplementary Figure 1 at http://www.BiochemJ.org/bj/406/bj4060057add.htm). To confirm that the growth factor secreted by BT-16 cells corresponded to human insulin, supernatants of BT-16 cells were analysed by MS. MS analysis revealed the presence of a protein of 5807 Da, corresponding to human insulin in serum-free supernatants obtained from BT-16 cells (Figure 2B). Immunofluorescence analysis revealed that individual BT-16 cells co-expressed insulin and the IR, indicating the presence of an autocrine signalling loop in these cells (Figure 2C). We next performed RT–PCR analysis to investigate the expression of the transcription factor PDX-1 in the panel of cell lines. PDX-1 is a master regulator of pancreas development and β-cell function, which participates in the transcription of several genes, including *insulin* [44]. This analysis revealed strong expression of PDX-1 mRNA in the BT-12, BT-16 and MRT cell lines, confirming their

insulin-producing phenotype (Figure 2D). We next investigated whether other human cancer cell lines also secrete insulin under similar culture conditions. However, an analysis of a panel of human medulloblastoma and neuroblastoma cell lines failed to document insulin secretion under serum-free culture conditions (results not shown), indicating that the response is selective for CNS AT/RT cells.

Inhibition of AT/RT cell proliferation by targeting the IR

The specific IGFIR/IR kinase inhibitor NVP-AEW541 [30] inhibited proliferation of BT-12 and BT-16 cell lines (Figure 3A), in a dose-dependent manner with IC_{50} values of 2 μM in BT-12 and 5 μM in BT-16 cells (Figure 3A). In BT-12 cells, NVP-AEW541 was effective at slightly lower concentrations, when cells were incubated in medium containing low serum, as compared with high serum (Figure 3A). In contrast, no differences in sensitivities were observed in the BT-16 cell line (Figure 3A). To confirm these observations, cell proliferation of BT-12 or BT-16 cells was assessed in the presence of neutralizing antibodies specific either for the IGFIR or for the IR. Proliferation of BT-16 cells in serum-free medium was significantly inhibited by the anti-IR neutralizing antibody (Figure 3B). A similar effect was observed with the anti-IGFIR neutralizing antibody (Figure 3B). The anti-IR neutralizing antibody also significantly inhibited proliferation of BT-12 and BT-16 cells in serum-containing medium (results not shown). Increasing concentrations of insulin stimulated proliferation of BT-16 cells in serum-free medium (Figure 3C) and the response was completely inhibited by a pharmacological PI3K inhibitor (LY294002), indicating that PI3K is essential for insulin-stimulated cellular responses in AT/RT cells. The maximal increase in cell proliferation was observed at an insulin concentration of 50 ng/ml (8.6 nM) and concentrations up to 1000 ng/ml had no significantly higher effect (Figure 3C). Inhibiting IGFIR/IR function with NVP-AEW541 also induced apoptosis in AT/RT cells, as assessed by caspase 3 activation (Figure 3D), indicating that the autocrine signalling loop involving the IR contributes to cell survival.

An RNAi approach was used to confirm the critical role of the IR and IGFIR in supporting AT/RT cell growth and survival. Transfection of BT-12 and BT-16 cells with siRNA targeting either the IRβ or the IGFIRβ resulted in a significant decrease in cell viability (Figures 4A and 4B and results not shown). To verify the specificity of the effect of the siRNA targeting the IRβ, protein down-regulation was assessed by Western-blot analysis in BT-12 and BT-16 cells (Figure 4C and results not shown).

Activation of the PI3K/Akt signalling pathway by insulin in AT/RT cells

The ability of polypeptide growth factors to activate PI3K/Akt, ERK1/2 and ribosomal protein S6K was then investigated in BT-12 and BT-16 cells. Insulin most potently activated Akt, as compared with EGF and SCF (stem cell factor), whereas activation of ERK1/2 was comparable between all growth factors (Figure 5A). The IGFIR/IR kinase inhibitor NVP-AEW541 (2.5 μM) completely inhibited IGF-I- and insulin-activated responses (Figure 5B). Autophosphorylation of the IR was also detected in insulin-stimulated AT/RT cells, confirming the specificity of the responses observed (Figure 5C).

Our data so far had revealed activation of the PI3K/Akt, S6K and ERK1/2 pathways in AT/RT cells stimulated by polypeptide growth factors. Therefore we next investigated the contributions of these pathways to proliferation in BT-12 and BT-16 cells.

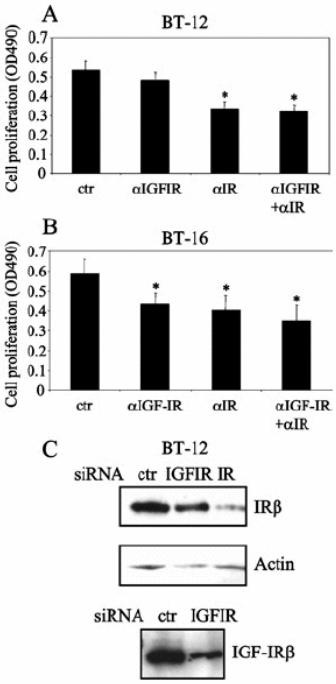

Figure 4 Down-regulation of IR by siRNA treatment impairs AT/RT cell proliferation

(**A**, **B**) BT-12 or BT-16 AT/RT cells were transiently transfected with non-targeting siRNA (ctr), or siRNA targeting the IGFIR or the IR, alone or in combination, where indicated. Cell proliferation was measured after 72 h. Results are the means with S.D. from three experiments performed in quadruplicate (*, $P < 0.05$ by ANOVA test). (**C**) BT-12 AT/RT cells were transiently transfected with siRNAs as indicated and expression of the IRβ and IGFIRβ was analysed by Western blot after 72 h.

Cell proliferation was assessed in the presence of the PI3K inhibitor LY294002, rapamycin, the MEK [MAPK (mitogen-activated protein kinase)/ERK kinase] inhibitor PD98059, or a pharmacological Akt/PKB inhibitor. AT/RT cell proliferation was significantly impaired by LY294002 or rapamycin, in both BT-12 and BT-16 cells grown in low (1%) or high (10%) serum (Figure 5D). The Akt inhibitor completely inhibited proliferation of the BT-12 cell line in low, but not high serum, while having a lesser effect in the BT-16 cell line (Figure 5D). To confirm the involvement of PI3K/Akt signalling in AT/RT cell proliferation, a dominant-negative catalytically inactive Akt (AktDN) construct was transiently transfected into BT-12 and BT-16 cells. Western-blot analysis confirmed expression of the construct in AT/RT cells (results not shown). The AktDN construct significantly inhibited proliferation in both cell lines (Figure 5E). In contrast, transfection of an activated Akt mutant (myrAktCA) had no significant effect on AT/RT cell proliferation.

The class I_A PI3K isoform p110α controls AT/RT cell proliferation and insulin-stimulated Akt activation

To confirm the involvement of PI3K signalling in AT/RT cell responses to insulin, a panel of isoform-specific class I_A PI3K inhibitors was tested in AT/RT cells. The pharmacological p110α inhibitor YM024 [45] inhibited proliferation of AT/RT cells in a dose-dependent manner with an IC_{50} of 1.5 μM (Figure 6A). In contrast, the p110β inhibitor TGX-221 [46] and the p110δ

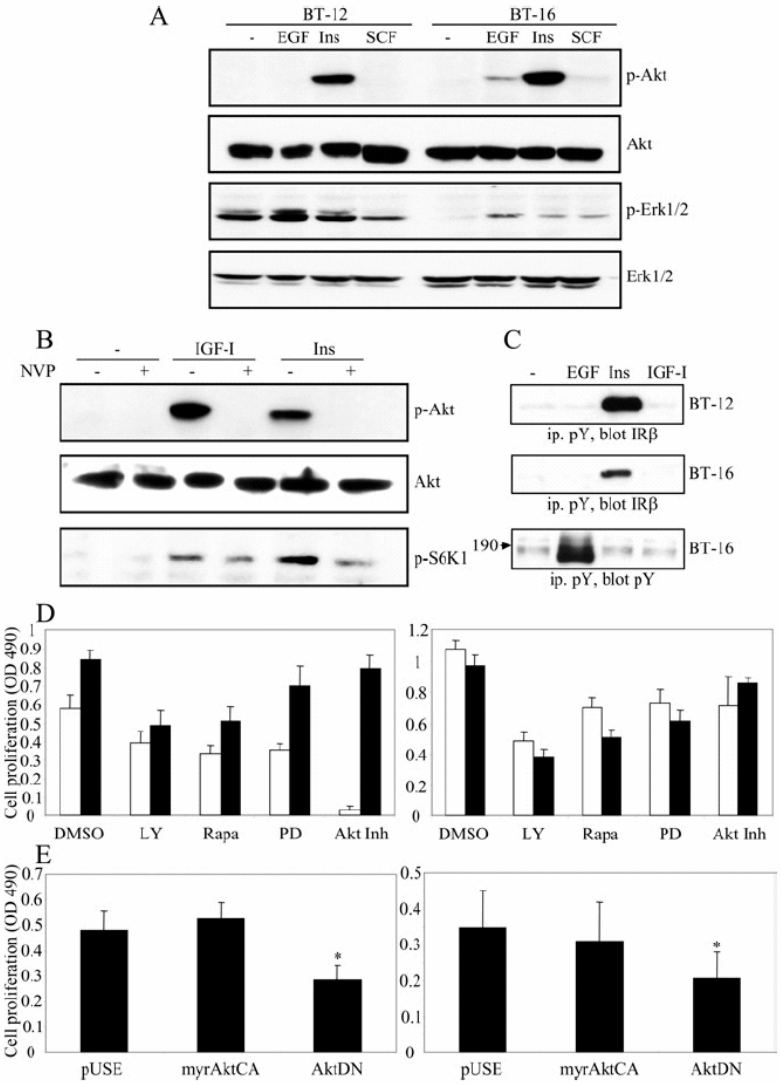

Figure 5 Insulin activates the PI3K/Akt pathway in AT/RT cells

(**A**) Serum-starved AT/RT cells were stimulated with growth factors where indicated (40 ng/ml EGF; 50 ng/ml Ins; 20 ng/ml SCF) for 10 min, and activation of Akt and ERK was determined by Western blot with phospho-specific antibodies. Abbreviations: p-Akt, Ser^{473}-phosphorylated Akt; p-ERK, Thr^{202}/Tyr^{204}-phosphorylated ERK. (**B**) Serum-starved BT-16 cells were pretreated with NVP-AEW541 (2.5 μM), stimulated with the growth factors indicated (50 ng/ml) for 10 min and activation of Akt and S6K1 was determined as above. Abbreviation: p-S6K1, Thr^{389}-phosphorylated S6K. (**C**) Serum-starved AT/RT cells were stimulated with the growth factors indicated (10 min) and anti-phosphotyrosine (pY) immunoprecipitates were analysed by Western blots for the relevant receptors. (**D**) BT-12 (left panel) or BT-16 (right panel) cells were incubated in a medium containing 10 % (closed bars) or 0.5 % (open bars) FCS in the presence of the inhibitors indicated [LY294002 10 μM (LY); rapamycin 20 ng/ml (Rapa); PD98059 25 μM (PD); Akt inhibitor 20 μM (Akt Inh)], or vehicle (DMSO). Cell proliferation was determined after 72 h. Results are the means with S.D. from a representative experiment (out of three) performed with eight repetitions. (**E**) BT-12 (left panel) or BT-16 (right panel) cells were transiently transfected with empty vector, or constructs encoding activated (myrAktCA) or dominant-negative Akt (AktDN). Cell proliferation was determined after 72 h. Results are the means with S.D. from three experiments performed in quadruplicate (*, $P < 0.05$ by ANOVA test).

inhibitor IC87114 [47] had a less pronounced effect on AT/RT cell proliferation (Figure 6A). The pharmacological p110α inhibitor YM024 also inhibited insulin-stimulated Akt activation, whereas TGX-221 and IC87114 did not impair the response (Figure 6B and results not shown). shRNA constructs targeting the catalytic subunits of class I_A PI3Ks were then transiently transfected into BT-16 cells to investigate their impact on cell proliferation. Western-blot analysis confirmed down-regulation of the expression of the target genes by the relevant shRNA construct (Figure 6D). The shRNA constructs targeting *PIK3CA* (encoding p110α) strongly (>80 %) inhibited BT-16 cell proliferation (Figure 6C). In contrast, RNAi targeting of *PIK3CB* resulted in no significant effect on AT/RT cell proliferation (Figure 6C). Moreover, insulin-stimulated Akt activation was abrogated by transfection of BT-16 cells with the shRNA construct targeting *PIK3CA* (results not shown). Thus the p110α isoform appears to play a crucial role in controlling AT/RT cell proliferation and insulin-stimulated Akt activation.

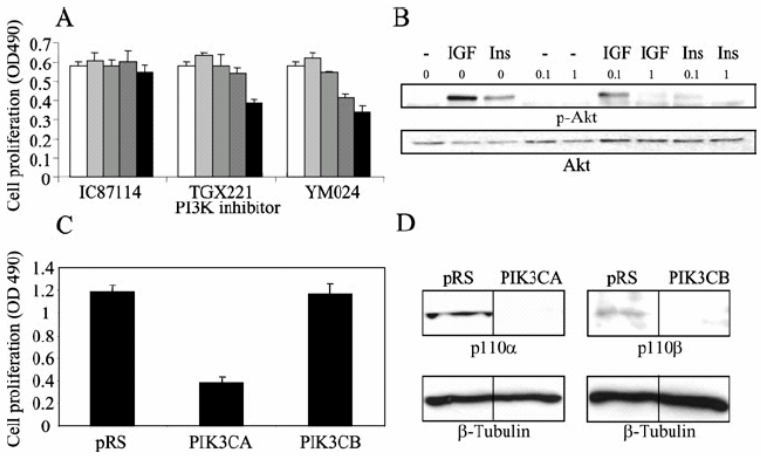

Figure 6 The PI3K p110α isoform controls AT/RT cell proliferation and insulin signalling

(**A**) BT-16 cells were incubated with increasing concentrations of isoform-specific PI3K inhibitors [0.01 μM (light grey bars), 0.1 μM (medium grey bars), 1 μM (dark grey bars), 10 μM (closed bars) or vehicle (open bars)]. Cell proliferation was determined after 72 h. Results are the means with S.D. from a representative experiment (out of three) performed in quadruplicate. (**B**) Serum-starved BT-16 cells were pre-incubated with the p110α inhibitor YM024 (micromolar concentrations) and stimulated with growth factors (50 ng/ml insulin; 50 ng/ml IGF-I) where indicated for 10 min. Akt activation was determined by Western blot with phospho-specific antibodies. Abbreviation: p-Akt, Ser^{473}-phosphorylated Akt. (**C**, **D**) BT-16 cells were transiently transfected with shRNA constructs targeting PI3K isoforms (PIK3CA: p110α; PIK3CB: p110β) or empty vector (pRS). (**C**) Cell proliferation was assessed after 72 h. Results are the means with S.D. from a representative experiment (out of three) performed with eight repetitions. (**D**) Protein down-regulation was determined by Western-blot analysis.

Ectopic expression of INI1 does not affect the IR signalling pathway in AT/RT cells

Since AT/RT cells frequently display mutations in the *INI1* gene, we investigated the impact of ectopic re-expression of the protein in BT-16 cells. BT-16 stably transfected with an INI1 expression vector displayed reduced proliferation under either low- or high-serum culture conditions (Figure 7A). However, BT-16 transfected with INI1 still displayed detectable insulin mRNA expression (Figure 7C). Moreover, the expression levels of the IR and IGFIR were not affected, as assessed by Western-blot analysis (Figure 7D). These results indicate that the establishment of the autocrine loop involving insulin and its receptor is independent of *INI1* loss in AT/RT cells.

DISCUSSION

Molecular abnormalities linked with human AT/RT include germline and somatic mutations in the *INI1* gene that encodes a chromatin remodelling factor and has been suggested to function as a tumour suppressor [7–9]. A recent report has also documented epigenetic repression of the *RASSF1* (Ras association domain family 1) gene in AT/RT cell lines and primary tumours [41]. RTK signalling has not yet been studied in detail in AT/RT cells. Expression of the IGFIR and IGF-II was reported in a limited number of cases of AT/RT tumours [25,48]. Targeting the IGFIR with antisense oligonucleotides resulted in increased apoptosis and sensitivity to the chemotherapeutic agents cisplatin and doxorubicin in AT/RT cells [32]. In the case of the MRTs, the EGFR inhibitor gefitinib was reported to have anti-tumour effects *in vitro* and *in vivo* [49]. Moreover, the PI3K/Akt pathway was reported to be involved in the resistance of MRT cells to chemotherapy and radiotherapy [50].

We have characterized a panel of human MRT and AT/RT cell lines for expression of RTKs and their downstream signalling mediators, in order to study the potential of targeting these molecules to inhibit cell proliferation and survival. The IR and

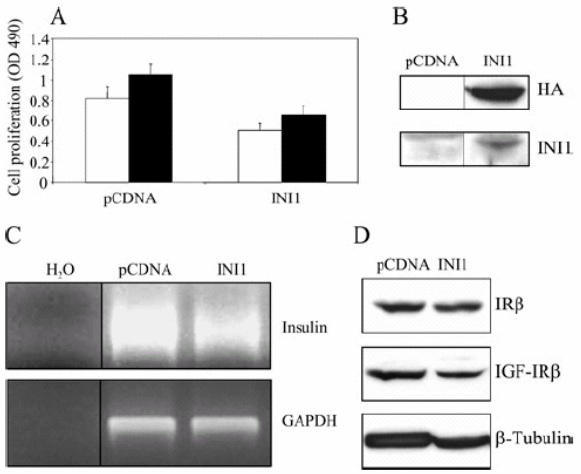

Figure 7 Ectopic expression of *INI1* reduces the proliferation of AT/RT cells, without effect on IR expression

(**A**) BT-16 cells were stably transfected with empty vector (pcDNA) or HA-tagged INI1. Cell proliferation in a medium containing 0.5% (open bars) or 10% (closed bars) FCS was determined after 72 h. Results are the means with S.D. from a representative experiment (out of three) performed with eight repetitions. (**B**) Expression of HA-tagged INI1 in the transfected BT-16 cells was verified by Western-blot analysis. (**C**) Insulin mRNA expression in BT-16 cells transfected with empty vector (pcDNA) or HA-tagged INI1 was determined by RT–PCR. GAPDH was analysed in parallel as a control. (**D**) The expression of the IRβ and the IGFIRβ in BT-16 cells transfected with empty vector (pcDNA) or HA-tagged INI1 was determined by Western blot.

IGFIR were found overexpressed in both AT/RT and MRT cell lines, as compared with normal brain tissue. Moreover and surprisingly, AT/RT cell lines secreted insulin when grown under serum-free conditions, indicating autocrine signalling events. To our knowledge these AT/RT cell lines are the first example of human CNS tumour cell lines secreting insulin. Moreover, the

only other examples of insulin secretion by cells other than pancreatic β-cells are sperm cells [51]. The concentration of insulin measured in supernatants from BT-16 cells was 2.5 nM, which is in the biological range. Maximal biological responsiveness to insulin tends to occur at concentrations in the range 10–20 nM [52–54] and insulin had maximal effects on BT-16 cell proliferation at a concentration of 8.6 nM. It is conceivable that AT/RT cells also produce other growth factors in an autocrine fashion. Indeed, IGF-I and IGF-II were detected at the mRNA level in BT-16 and BT-12 cells respectively, as recently reported [32]. We were unable to detect free IGF-I and IGF-II in supernatants from AT/RT cells by ELISA, but this could be due to the fact that IGFs were bound to IGF-binding proteins. Autocrine signalling by IGF-I via the IGFIR in BT-16 cells would explain the inhibitory effects of IGFIR-neutralizing antibodies or siRNA on the proliferation of these cells.

Insulin activated PI3K/Akt, S6K and ERK signalling, whereas other growth factors such as EGF or SCF selectively activated some, but not all of the pathways. Targeting the IGFIR kinase activity with NVP-AEW541 resulted in inhibition of AT/RT proliferation, although at higher IC_{50} values than reported in other tumour cells [30]. These increased IC_{50} values possibly reflect co-expression of the IR and IGFIR in AT/RT cell lines, since the IC_{50} of NVP-AEW541 for the IR was reported to be 2.3 μM in cells [30,55]. This model was supported by the observations that neutralizing antibodies or siRNA targeting the IRβ inhibited the proliferation of AT/RT cells. Consequently, our results demonstrate that insulin secreted by AT/RT cells in an autocrine fashion activates the IR and thus contributes to AT/RT cell proliferation and survival. This mechanism of IR activation appears to be different from the previously reported activation loop involving IGF-II and the IR isoform A in human cancer cells [56–58]. The molecular mechanisms underlying the establishment of the autocrine insulin signalling loop in AT/RT cells are currently unclear. Loss of *hSNF5/INI1* did not appear to be a major cause of the expression of insulin and the IR by AT/RT cells, since transfection of INI1 did not substantially alter the expression of these molecules. Intriguingly, the pancreatic transcription factor PDX-1 was detected in AT/RT and MRT cells, indicating that aberrant expression of transcription factors may drive these cancer cells towards an insulin-producing phenotype. A recent survey of mutations in human tumour genomes has revealed frequent mutations in transcription factors, emphasizing the contribution of abnormalities in transcription to the development of human cancer [59].

To investigate whether targeting downstream signalling mediators of the IR could provide a means of inhibiting proliferation of AT/RT cells, we used a combination of pharmacological and RNAi approaches. This revealed that the PI3K/Akt/mTOR pathway was crucial in the control of AT/RT cell proliferation. Moreover, a selective role for the PI3K isoform p110α in transducing signals from the IR was uncovered in AT/RT cells, in good agreement with recent reports documenting the crucial role of p110α in insulin signalling [60,61]. Thus pharmacological inhibitors targeting the PI3K p110α isoform may have antiproliferative potential in human cancer cells where this isoform is activated by the IR.

Insulin has various biological effects in a broad range of tissues, and its functions in the brain include neuronal survival and regulation of energy homoeostasis [62]. Although a large body of evidence exists implicating IGF-I and IGF-II as autocrine growth factors in a broad range of human malignancies [24,27], such a function has not yet been described for insulin in CNS tumours. In the light of the novel function for insulin as an autocrine growth factor for CNS AT/RT cells, further studies are warranted to uncover its potential role in other human cancers.

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REFERENCES

1 Biegel, J. A., Tan, L., Zhang, F., Wainwright, L., Russo, P. and Rorke, L. B. (2002) Alterations of the hSNF5/INI1 gene in central nervous system atypical teratoid/rhabdoid tumors and renal and extrarenal rhabdoid tumors. Clin. Cancer Res. **8**, 3461–3467

2 Judkins, A. R., Mauger, J., Ht, A., Rorke, L. B. and Biegel, J. A. (2004) Immunohistochemical analysis of hSNF5/INI1 in pediatric CNS neoplasms. Am. J. Surg. Pathol. **28**, 644–650

3 Pomeroy, S. L., Tamayo, P., Gaasenbeek, M., Sturla, L. M., Angelo, M., McLaughlin, M. E., Kim, J. Y., Goumnerova, L. C., Black, P. M., Lau, C. et al. (2002) Prediction of central nervous system embryonal tumour outcome based on gene expression. Nature **415**, 436–442

4 Reddy, A. T. (2005) A typical teratoid/rhabdoid tumors of the central nervous system. J. Neurooncol. **75**, 309–313

5 Hilden, J. M., Meerbaum, S., Burger, P., Finlay, J., Janss, A., Scheithauer, B. W., Walter, A. W., Rorke, L. B. and Biegel, J. A. (2004) Central nervous system atypical teratoid/rhabdoid tumor: results of therapy in children enrolled in a registry. J. Clin. Oncol. **22**, 2877–2884

6 Tekautz, T. M., Fuller, C. E., Blaney, S., Fouladi, M., Broniscer, A., Merchant, T. E., Krasin, M., Dalton, J., Hale, G., Kun, L. E. et al. (2005) A typical teratoid/rhabdoid tumors (ATRT): improved survival in children 3 years of age and older with radiation therapy and high-dose alkylator-based chemotherapy. J. Clin. Oncol. **23**, 1491–1499

7 Biegel, J. A., Zhou, J. Y., Rorke, L. B., Stenstrom, C., Wainwright, L. M. and Fogelgren, B. (1999) Germ-line and acquired mutations of INI1 in atypical teratoid and rhabdoid tumors. Cancer Res. **59**, 74–79

8 Sevenet, N., Lellouch-Tubiana, A., Schofield, D., Hoang-Xuan, K., Gessler, M., Birnbaum, D., Jeanpierre, C., Jouvet, A. and Delattre, O. (1999) Spectrum of hSNF5/INI1 somatic mutations in human cancer and genotype–phenotype correlations. Hum. Mol. Genet. **8**, 2359–2368

9 Versteege, I., Sevenet, N., Lange, J., Rousseau-Merck, M. F., Ambros, P., Handgretinger, R., Aurias, A. and Delattre, O. (1998) Truncating mutations of hSNF5/INI1 in aggressive paediatric cancer. Nature **394**, 203–206

10 Wang, W., Cote, J., Xue, Y., Zhou, S., Khavari, P. A., Biggar, S. R., Muchardt, C., Kalpana, G. V., Goff, S. P., Yaniv, M. et al. (1996) Purification and biochemical heterogeneity of the mammalian SWI–SNF complex. EMBO J. **15**, 5370–5382

11 Wang, W., Xue, Y., Zhou, S., Kuo, A., Cairns, B. R. and Crabtree, G. R. (1996) Diversity and specialization of mammalian SWI/SNF complexes. Genes Dev. **10**, 2117–2130

12 Roberts, C. W. and Orkin, S. H. (2004) The SWI/SNF complex – chromatin and cancer. Nat. Rev. Cancer **4**, 133–142

13 Guidi, C. J., Sands, A. T., Zambrowicz, B. P., Turner, T. K., Demers, D. A., Webster, W., Smith, T. W., Imbalzano, A. N. and Jones, S. N. (2001) Disruption of Ini1 leads to peri-implantation lethality and tumorigenesis in mice. Mol. Cell. Biol. **21**, 3598–3603

14 Roberts, C. W., Galusha, S. A., McMenamin, M. E., Fletcher, C. D. and Orkin, S. H. (2000) Haploinsufficiency of Snf5 (integrase interactor 1) predisposes to malignant rhabdoid tumors in mice. Proc. Natl. Acad. Sci. U.S.A. **97**, 13796–13800

15 Isakoff, M. S., Sansam, C. G., Tamayo, P., Subramanian, A., Evans, J. A., Fillmore, C. M., Wang, X., Biegel, J. A., Pomeroy, S. L., Mesirov, J. P. and Roberts, C. W. (2005) Inactivation of the Snf5 tumor suppressor stimulates cell cycle progression and cooperates with p53 loss in oncogenic transformation. Proc. Natl. Acad. Sci. U.S.A. **102**, 17745–17750

16 Klochendler-Yeivin, A., Picarsky, E. and Yaniv, M. (2006) Increased DNA damage sensitivity and apoptosis in cells lacking the Snf5/Ini1 subunit of the SWI/SNF chromatin remodeling complex. Mol. Cell. Biol. **26**, 2661–2674

17 Zhang, Z. K., Davies, K. P., Allen, J., Zhu, L., Pestell, R. G., Zagzag, D. and Kalpana, G. V. (2002) Cell cycle arrest and repression of cyclin D1 transcription by INI1/hSNF5. Mol. Cell. Biol. **22**, 5975–5988

18 Alarcon-Vargas, D., Zhang, Z., Agarwal, B., Challagulla, K., Mani, S. and Kalpana, G. V. (2006) Targeting cyclin D1, a downstream effector of INI1/hSNF5, in rhabdoid tumors. Oncogene **25**, 722–734
19 Jones, J. I. and Clemmons, D. R. (1995) Insulin-like growth factors and their binding proteins: biological actions. Endocr. Rev. **16**, 3–34
20 Denley, A., Cosgrove, L. J., Booker, G. W., Wallace, J. C. and Forbes, B. E. (2005) Molecular interactions of the IGF system. Cytokine Growth Factor Rev. **16**, 421–439
21 Kornfeld, S. (1992) Structure and function of the mannose 6-phosphate/insulin like growth factor II receptors. Annu. Rev. Biochem. **61**, 307–330
22 Hebert, E. (2006) Mannose-6-phosphate/insulin-like growth factor II receptor expression and tumor development. Biosci. Rep. **26**, 7–17
23 Foulstone, E., Prince, S., Zaccheo, O., Burns, J. L., Harper, J., Jacobs, C., Church, D. and Hassan, A. B. (2005) Insulin-like growth factor ligands, receptors, and binding proteins in cancer. J. Pathol. **205**, 145–153
24 Pollak, M. N., Schernhammer, E. S. and Hankinson, S. E. (2004) Insulin-like growth factors and neoplasia. Nat. Rev. Cancer **4**, 505–518
25 Ogino, S., Kubo, S., Abdul-Karim, F. W. and Cohen, M. L. (2001) Comparative immunohistochemical study of insulin-like growth factor II and insulin-like growth factor receptor type 1 in pediatric brain tumors. Pediatr. Dev. Pathol. **4**, 23–31
26 Surmacz, E. (2003) Growth factor receptors as therapeutic targets: strategies to inhibit the insulin-like growth factor I receptor. Oncogene **22**, 6589–6597
27 Guerreiro, A. S., Boller, D., Doepfner, K. T. and Arcaro, A. (2006) IGF-IR: potential role in antitumor agents. Drug News Perspect. **19**, 261–272
28 Yee, D. (2006) Targeting insulin-like growth factor pathways. Br. J. Cancer **94**, 465–468
29 Miller, B. S. and Yee, D. (2005) Type I insulin-like growth factor receptor as a therapeutic target in cancer. Cancer Res. **65**, 10123–10127
30 Garcia-Echeverria, C., Pearson, M. A., Marti, A., Meyer, T., Mestan, J., Zimmermann, J., Gao, J., Brueggen, J., Capraro, H. G., Cozens, R. et al. (2004) *In vivo* antitumor activity of NVP-AEW541 – a novel, potent, and selective inhibitor of the IGF-IR kinase. Cancer Cell **5**, 231–239
31 Urbanska, K., Trojanek, J., Del Valle, L., Eldeen, M. B., Hofmann, F., Garcia-Echeverria, C., Khalili, K. and Reiss, K. (2007) Inhibition of IGF-I receptor in anchorage-independence attenuates GSK-3beta constitutive phosphorylation and compromises growth and survival of medulloblastoma cell lines. Oncogene **26**, 2308–2317
32 D'Cunja, J., Shalaby, T., Rivera, P., von Buren, A., Patti, R., Heppner, F. L., Arcaro, A., Rorke-Adams, L. B., Phillips, P. C. and Grotzer, M. A. (2007) Antisense treatment of IGF-IR induces apoptosis and enhances chemosensitivity in central nervous system atypical teratoid/rhabdoid tumours cells. Eur. J. Cancer **43**, 1581–1589
33 Denley, A., Wallace, J. C., Cosgrove, L. J. and Forbes, B. E. (2003) The insulin receptor isoform exon 11- (IR-A) in cancer and other diseases: a review. Horm. Metab. Res. **35**, 778–785
34 Vanhaesebroeck, B., Leevers, S. J., Panayotou, G. and Waterfield, M. D. (1997) Phosphoinositide 3-kinases: a conserved family of signal transducers. Trends Biochem. Sci. **22**, 267–272
35 Vanhaesebroeck, B., Leevers, S. J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P. C., Woscholski, R., Parker, P. J. and Waterfield, M. D. (2001) Synthesis and function of 3-phosphorylated inositol lipids. Annu. Rev. Biochem. **70**, 535–602
36 Katso, R., Okkenhaug, K., Ahmadi, K., White, S., Timms, J. and Waterfield, M. D. (2001) Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. Annu. Rev. Cell Dev. Biol. **17**, 615–675
37 Cantley, L. C. and Neel, B. G. (1999) New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc. Natl. Acad. Sci. U.S.A. **96**, 4240–4245
38 Maehama, T., Taylor, G. S. and Dixon, J. E. (2001) PTEN and myotubularin: novel phosphoinositide phosphatases. Annu. Rev. Biochem. **70**, 247–279
39 Samuels, Y., Wang, Z., Bardelli, A., Silliman, N., Ptak, J., Szabo, S., Yan, H., Gazdar, A., Powell, S. M., Riggins, G. J. et al. (2004) High frequency of mutations of the PIK3CA gene in human cancers. Science **304**, 554
40 Broderick, D. K., Di, C., Parrett, T. J., Samuels, Y. R., Cummins, J. M., McLendon, R. E., Fults, D. W., Velculescu, V. E., Bigner, D. D. and Yan, H. (2004) Mutations of PIK3CA in anaplastic oligodendrogliomas, high-grade astrocytomas, and medulloblastomas. Cancer Res. **64**, 5048–5050
41 Muhlisch, J., Schwering, A., Grotzer, M., Vince, G. H., Roggendorf, W., Hagemann, C., Sorensen, N., Rickert, C. H., Osada, N., Jurgens, H. and Fruhwald, M. C. (2006) Epigenetic repression of RASSF1A but not CASP8 in supratentorial PNET (sPNET) and atypical teratoid/rhabdoid tumors (AT/RT) of childhood. Oncogene **25**, 1111–1117
42 Paddison, P. J., Silva, J. M., Conklin, D. S., Schlabach, M., Li, M., Aruleba, S., Balija, V., O'Shaughnessy, A., Gnoj, L., Scobie, K. et al. (2004) A resource for large-scale RNA-interference-based screens in mammals. Nature **428**, 427–431
43 Biegel, J. A., Fogelgren, B., Zhou, J. Y., James, C. D., Janss, A. J., Allen, J. C., Zagzag, D., Raffel, C. and Rorke, L. B. (2000) Mutations of the INI1 rhabdoid tumor suppressor gene in medulloblastomas and primitive neuroectodermal tumors of the central nervous system. Clin. Cancer Res. **6**, 2759–2763
44 Ohlsson, H., Karlsson, K. and Edlund, T. (1993) IPF1, a homeodomain-containing transactivator of the insulin gene. EMBO J. **12**, 4251–4259
45 Condliffe, A. M., Davidson, K., Anderson, K. E., Ellson, C. D., Crabbe, T., Okkenhaug, K., Vanhaesebroeck, B., Turner, M., Webb, L., Wymann, M. P. et al. (2005) Sequential activation of class IB and class IA PI3K is important for the primed respiratory burst of human but not murine neutrophils. Blood **106**, 1432–1440
46 Jackson, S. P., Schoenwaelder, S. M., Goncalves, I., Nesbitt, W. S., Yap, C. L., Wright, C. E., Kenche, V., Anderson, K. E., Dopheide, S. M., Yuan, Y. et al. (2005) PI 3-kinase p110beta: a new target for antithrombotic therapy. Nat. Med. **11**, 507–514
47 Sadhu, C., Masinovsky, B., Dick, K., Sowell, C. G. and Staunton, D. E. (2003) Essential role of phosphoinositide 3-kinase delta in neutrophil directional movement. J. Immunol. **170**, 2647–2654
48 Ogino, S., Cohen, M. L. and Abdul-Karim, F. W. (1999) Atypical teratoid/rhabdoid tumor of the CNS: cytopathology and immunohistochemistry of insulin-like growth factor-II, insulin-like growth factor receptor type 1, cathepsin D, and Ki-67. Mod. Pathol. **12**, 379–385
49 Kuwahara, Y., Hosoi, H., Osone, S., Kita, M., Iehara, T., Kuroda, H. and Sugimoto, T. (2004) Antitumor activity of gefitinib in malignant rhabdoid tumor cells *in vitro* and *in vivo*. Clin. Cancer Res. **10**, 5940–5948
50 Nocentini, S. (2003) Apoptotic response of malignant rhabdoid tumor cells. Cancer Cell Int. **3**, 11
51 Aquila, S., Gentile, M., Middea, E., Catalano, S. and Ando, S. (2005) Autocrine regulation of insulin secretion in human ejaculated spermatozoa. Endocrinology **146**, 552–557
52 Kern, M., Wells, J. A., Stephens, J. M., Elton, C. W., Friedman, J. E., Tapscott, E. B., Pekala, P. H. and Dohm, G. L. (1990) Insulin responsiveness in skeletal muscle is determined by glucose transporter (Glut4) protein level. Biochem. J. **270**, 397–400
53 Desbois, C., Capeau, J., Hainault, I., Wicek, D., Reynet, C., Veissiere, D., Caron, M., Picard, J., Guerre-Millo, M. and Cherqui, G. (1992) Differential role of insulin receptor autophosphorylation sites 1162 and 1163 in the long-term insulin stimulation of glucose transport, glycogenesis, and protein synthesis. J. Biol. Chem. **267**, 13488–13497
54 Wong, E. H., Tan, C. H., Khoo, H. E., Ng, F. H., Lim, K. L. and Ciaraldi, T. P. (1995) Rat fibroblast cells overexpressing kinase-inactive human insulin receptors are insulin responsive: influence of growth conditions. Endocrinology **136**, 1459–1467
55 Guerreiro, A. S., Boller, D., Shalaby, T., Grotzer, M. A. and Arcaro, A. (2006) Protein kinase B modulates the sensitivity of human neuroblastoma cells to insulin-like growth factor receptor inhibition. Int. J. Cancer. **119**, 2527–2538
56 Frasca, F., Pandini, G., Scalia, P., Sciacca, L., Mineo, R., Costantino, A., Goldfine, I. D., Belfiore, A. and Vigneri, R. (1999) Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. Mol. Cell. Biol. **19**, 3278–3288
57 Sciacca, L., Costantino, A., Pandini, G., Mineo, R., Frasca, F., Scalia, P., Sbraccia, P., Goldfine, I. D., Vigneri, R. and Belfiore, A. (1999) Insulin receptor activation by IGF-II in breast cancers: evidence for a new autocrine/paracrine mechanism. Oncogene **18**, 2471–2479
58 Morrione, A., Valentinis, B., Xu, S. Q., Yumet, G., Louvi, A., Efstratiadis, A. and Baserga, R. (1997) Insulin-like growth factor II stimulates cell proliferation through the insulin receptor. Proc. Natl. Acad. Sci. U.S.A. **94**, 3777–3782
59 Sjoblom, T., Jones, S., Wood, L. D., Parsons, D. W., Lin, J., Barber, T. D., Mandelker, D., Leary, R. J., Ptak, J., Silliman, N. et al. (2006) The consensus coding sequences of human breast and colorectal cancers. Science **314**, 268–274
60 Foukas, L. C., Claret, M., Pearce, W., Okkenhaug, K., Meek, S., Peskett, E., Sancho, S., Smith, A. J., Withers, D. J. and Vanhaesebroeck, B. (2006) Critical role for the p110alpha phosphoinositide-3-OH kinase in growth and metabolic regulation. Nature **441**, 366–370
61 Knight, Z. A., Gonzalez, B., Feldman, M. E., Zunder, E. R., Goldenberg, D. D., Williams, O., Loewith, R., Stokoe, D., Balla, A., Toth, B. et al. (2006) A pharmacological map of the PI3-K family defines a role for p110alpha in insulin signaling. Cell **125**, 733–747
62 Plum, L., Belgardt, B. F. and Bruning, J. C. (2006) Central insulin action in energy and glucose homeostasis. J. Clin. Invest. **116**, 1761–1766

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3.6. Targeting the phosphoinositide 3-kinase isoform p110δ impairs growth and survival in neuroblastoma cells

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Targeting the phosphoinositide 3-kinase isoform p110δ impairs growth and survival in neuroblastoma cells

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Running title: Role of p110δ in neuroblastoma

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Abstract

Purpose: The phosphoinositide 3-kinase (PI3K)/Akt pathway is frequently activated in human cancer and plays a crucial role in neuroblastoma biology. We were interested in gaining further insight into the potential of targeting PI3K/Akt signalling as a novel anti-proliferative approach in neuroblastoma.

Experimental Design: The expression pattern and functions of class I_A PI3K isoforms were investigated in tumor samples and cell lines. Effects on cell survival and downstream signalling were analysed following down-regulation of p110α or p110δ in SH-SY5Y and LA-N-1 cells by means of RNA interference (RNAi).

Results: Over-expression of the catalytic p110δ and regulatory p85α isoforms was detected in a panel of primary neuroblastoma samples and cell lines, as compared to normal adrenal gland tissue. While down-regulation of either p110α or p110δ led to impaired cell growth, reduced expression of p110δ also had a selective effect on the survival of SH-SY5Y cells. Decreased levels of p110δ were found to induce apoptosis and lead to lower expression levels of anti-apoptotic Bcl-2 family proteins. SH-SY5Y cells with decreased p110δ levels also displayed reduced activation of ribosomal protein S6 kinase (S6K) in response to stimulation with epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1).

Conclusions: Together, our data reveal a novel function of p110δ in neuroblastoma growth and survival.

Introduction

Neuroblastoma is the most common extra cranial solid tumor occurring in children and accounts for 8% to 10% of all paediatric malignancies (1, 2). High-risk disease, present in about half of the patients, is characterised by unresectable primary lesions and metastasis (2). Treatment of high-risk neuroblastoma with high-dose chemotherapy including peripheral stem cell rescue and radiotherapy resulted only in 34% three-year event-free survival probability (3).

A better understanding of the biology of neuroblastoma will potentially lead to the identification of novel therapeutic targets, which in turn could facilitate the development of new drugs for neuroblastoma. A promising field of investigation is to target receptor tyrosine kinase (RTK) signalling to some of their downstream mediators such as phosphoinositide 3-kinase (PI3K), Akt and the mammalian target of rapamycin (mTOR). Polypeptide growth factors have indeed been shown to play a key role in neuroblastoma biology. Insulin-like growth factor (IGF) signalling has been extensively studied in the context of neuroblastoma proliferation, survival and motility (4-7). Several potential anti-tumor approaches involving the IGF-1 system have been reported in neuroblastoma (8, 9). Moreover, inhibition of platelet-derived growth factor receptor (PDGFR) and c-Kit signalling with imatinib mesylate was reported to impair growth in neuroblastoma cell lines (10). Neurotrophins such as brain-derived neurotrophic factor (BDNF) also play an important role in neuroblastoma chemoresistance by binding to the Trk receptor family (11).

In view of the fact that neuroblastoma express a variety of different RTKs, it remains unclear whether targeting individual receptors will provide a successful therapeutic strategy. An alternative approach would be to identify downstream signalling molecules essential for transmitting the proliferative and survival message of several different RTKs. Phosphoinositide 3-kinase (PI3K) represents such a molecule, in view of its crucial role in controlling cell proliferation, survival and motility/metastasis downstream of many different RTKs (12-14). PI3Ks are an enzyme family comprising eight catalytic isoforms in humans, with different substrate specificities, regulatory mechanisms and tissue distribution (12, 14). The importance of PI3K signalling in human cancer was first demonstrated by the observation that mutations in the tumor suppressor gene *PTEN* occur frequently in human tumours. PTEN is a phosphatase that antagonises the action of PI3K by dephosphorylating the D-3 position of polyphosphoinositides (14, 15). Moreover, recent reports have described activating mutations

in the *PIK3CA* gene encoding the catalytic p110α isoform of PI3K in a variety of human cancers, including breast, colon and ovarian cancer, as well as medulloblastoma (16). In neuroblastoma, brain-derived neurotrophic factor (BDNF) was shown to protect the tumor cells from chemotherapy-induced apoptosis via the PI3K pathway (17). IGF-1 signalling via PI3K was shown to be required for neuroblastoma differentiation, cytoskeletal rearrangements (18), as well as angiogenesis and vascular endothelial growth factor (VEGF) expression (19). PI3K signalling is also activated by epidermal growth factor (EGF) in neuroblastoma cells and contributes to cell proliferation by this growth factor (20). Thus, targeting the PI3K/Akt/mTOR/S6K pathway may represent an attractive novel approach to develop therapies for neuroblastoma.

In the present report we have evaluated the expression of PI3K isoforms in primary human neuroblastoma samples and cell lines. Moreover, we have investigated whether targeting distinct PI3K isoforms could impair growth and survival of neuroblastoma cell lines. Our results show for the first time that the class I_A PI3K p110δ is over-expressed in a subset of neuroblastoma samples and plays a crucial role in the growth and survival of neuroblastoma cells.

Materials and methods

Reagents and Antibodies. Antibodies were purchased from the following companies: p85α, p110β, p110δ, PARP, PTEN, Akt/PKB, Erk1/2, Santa Cruz Biotechnology; S6 protein, 4EBP1 and phosphospecific antibodies for Akt/PKB (Ser473; Thr308), Erk1/2 (Thr202/Tyr204), S6 protein (Ser235/236; Ser240/244), 4E-BP1 (Thr37/46), Cell Signaling Technology; β-Actin, Sigma-Aldrich; p110α (clone U3A), generous gift from Dr. A. Klippel. Analysis of proteins involved in apoptosis was performed using the Pro-Survival and the Pro-Apoptosis Bcl-2 Family Antibody Sampler Kit (Cell Signaling). LY294002, rapamycin, EGF, PDGF and IGF-1 were obtained from Calbiochem.

Determination of PI3K-related gene expression in human primary neuroblastoma samples by cDNA microarray analysis. Expression of PI3K-related genes was determined in tumours from 68 neuroblastoma patients by Affymetrix U95A array analysis. The patient cohort and data normalisation procedures have been described elsewhere (21). Correlation of PI3K-related gene expression with molecular and clinical parameters was determined using the stats package included in R2.2 (www.r-project.org). Visualisation of gene expression was accomplished using Spotfire 8.1.

Primary neuroblastoma samples. Ethical approval to use residual tissue was obtained. RNAlater-preserved tumor tissue was available from the Swiss Paediatric Oncology Group tumor bank from neuroblastoma patients diagnosed between January 2003 and December 2005 at the University Children's Hospital of Zurich, (n=14), the Children's Hospital Luzern (n=3), the University Children's Hospital Bern (n=1) and the University Children's Hospital Basel (n=1). The selection of the tumours for the study was based on the availability of a sufficient quantity of tumor tissue to perform RNA isolation. All diagnoses were confirmed by histological assessment of the tumor specimen obtained at surgery. An overview of the tumor characteristics is given in Supplemental Fig. 1.

Protein extraction from tumor samples. Tumor tissue was disrupted with a sterile disposable tissue grinder (Sage Products Inc.). Protein extracts were obtained using the PARIS Kit (Ambion) according to the manufacturer's instructions.

Isolation of RNA from tumor samples and RT-PCR. Tumor tissue was disrupted as described above and homogenised in guanidinium isothiocyanate-containing buffer. Total RNA was isolated using the RNeasy kit (Qiagen Inc.) according to the manufacturer's protocol. Total RNA (3 μg) from each tumor sample was converted into cDNA using the SuperScript™ First-Strand Synthesis System for PCR according to manufacturer's instructions (Invitrogen Life Technologies). mRNA expression of four target genes and 18S (internal control gene) was measured in tumor samples and cell lines by TaqMan® Assay-on-Demand™ Gene Expression products (Applied Biosystems). Normal human adrenal gland tissue (AG) was used as a reference. The following primers were used (gene – assay ID): PIK3R1 - Hs00236128_m1; PIK3CA - Hs00180679_m1; PIK3CB - Hs00178872_m1; PIK3CD - Hs00192399_m1; eukaryotic 18S rRNA - Hs99999901_s1. Three replicates were run for each sample in a 96-well format plate. Gene expression assays consisted of a FAM™ dye-labelled TaqMan® MGB probe and two PCR primers. The thermal cycling conditions consisted of an initial denaturation step at 95°C for 10 min and a 50-cycle countdown at 95°C for 15 s and 60°C for 1 min. Each sample was normalised on the basis of its 18S rRNA content. Relative mRNA expression levels were calculated using the comparative threshold cycle (CT) method (22).

Cell Culture. Human neuroblastoma cell lines were kindly provided by Dr Brodeur, Children's Hospital of Philadelphia. The cells were grown in RPMI (Life Technologies/Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS) and penicillin/streptomycin/L-glutamine and passaged every 3-5 days by trypsinization.

Stable Transfectants. SH-SY5Y and LA-N-1 cells were stably transfected with the murine ecotrophic receptor (EcoR) using Lipofectamine (Invitrogen) according to the manufacturer's protocol. 72 hours post transfection, cells were diluted in medium containing G418 (0.8 mg/ml). In parallel, retroviral plasmid constructs encoding short hairpin RNA (shRNA) specifically targeting p110α or p110δ (23) were transiently transfected into packing cells. The supernatant was diluted with 1 part RPMI containing G418 (final concentration: 0.8 mg/ml), as well as Polybrene (final concentration: 8μg/ml). The diluted supernatant was then added to cells stably expressing the EcoR. 24 hrs post infection, SH-SY5Y and LA-N-1 cells were split in medium containing puromycin (0.5 μg/ml). Single colonies were picked and

expanded in selective medium. Protein down-regulation was confirmed by Western blot analysis. All experiments were performed with two clones from each transfection.

Transient Transfection. Cells were transiently transfected using the Amaxa Nucleofector Device according to the optimised protocol provided for SH-SY5Y cells. The following constructs were used: pRS, pRS-PIK3CA, pRS-PIK3CD (23), pcDNA3 (Invitrogen) and pcDNA-S6K AK (9).

Cell Proliferation. Neuroblastoma cells were seeded in 96-well plates at a density of 7'500 cells/well and grown for 72 hrs in RPMI containing low (1%) or high (10%) serum. Alternatively, cells were treated with growth factors or inhibitors as indicated. Cell proliferation was analysed by the CellTiter 96® AQ_{ueous} Cell Proliferation Assay (Promega) according to the manufacturer's instructions.

Apoptosis. Neuroblastoma cells were seeded in 96-well plates at a density of 20'000 cells/well. Basal caspase-3/7 activity was measured after 24 hrs using the Caspase-Glo® 3/7 Assay (Promega) according to the manufacturer's instructions.

Cell Cycle Distribution. Cell cycle distribution was analysed by means of propidium iodide (PI) staining and Fluorescence-activated cell sorter (FACS) analysis. Cells were seeded in a 6-well plate at a density of 6.5 x 10^5 cells and incubated in RPMI containing low (1%) or high (10%) serum for 24 hrs. Cells were collected using 0.5% trypsin and resuspended in cold PBS. One-tenth volume of 10x propidium iodide solution (500 µg/ml propidium iodide, 10mg/ml sodium citrate and 1% (v/v) Triton X-100) was added and cells were acquired in a flow cytometer within half an hour.

Growth Factor Stimulations. Cells were grown to confluency in a 6-well plate and starved overnight in RPMI containing 0.5% FCS. Cells were maintained in serum-free RPMI for 1 hour in the presence or absence of inhibitors as indicated and were then stimulated with the indicated growth factors for 10min. Cellular lysates were prepared as described below.

Western blotting. Cellular lysates were prepared as described (24) and normalised using a bicinchoninic acid (BCA) protein assay (Pierce). Cell lysates were separated by

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a hydrophobic polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham Biosciences) and immunoblotted with the indicated antibodies prior to chemiluminescent detection (ECL Western blotting detection reagents; Amersham Biosciences).

Statistical Analysis. Spearman's rank correlation and the Exact Wilcoxon rank-sum test were used for the analysis of PI3K gene expression in primary neuroblastoma samples. For experiments on cell lines, the statistical significance of differences between groups was assessed with ANOVA using the Bonferroni multiple comparison test, $p < 0.01$ were indicated with a double asterisk.

Results

Over-expression of PI3K isoforms in primary human neuroblastoma samples and cell lines. To gain insight into the specific functions of class I_A PI3K isoforms in neuroblastoma, we initially assessed the expression levels of the regulatory and catalytic subunits in two independent groups of tumor samples. In a panel of 19 primary neuroblastoma samples, the expression levels of the class I_A PI3K regulatory and catalytic subunits were analysed by quantitative RT-PCR (qRT-PCR) (Fig. 1A). Expression of the *PIK3R1* (encoding the regulatory subunit p85α) and *PIK3CD* (encoding the catalytic subunit p110δ) genes was found to be increased more than 2-fold in 10/19 neuroblastoma samples when compared to normal adrenal gland tissue (Fig. 1A, left panel). In contrast, mRNA expression of the catalytic p110α and p110β isoforms was not increased (Fig. 1A, left panel). In this panel of tumor samples, expression of p85α and p110δ was significantly correlated (Spearman's rank correlation, $p<0.0001$), indicating that the p85α/p110δ heterodimer is over-expressed in neuroblastoma. Expression of p85α and p110δ was significantly higher in children under the age of one (median values: 2.48 for p85α and 5.17 for p110δ) than in patients older than one year of age (median values: 0.64 for p85α and 0.95 for p110δ) (Exact Wilcoxon rank-sum test, $p=0.0121$ for p85α; $p=0.0025$ for p110δ). Furthermore, the expression of p85α and p110δ was found to be significantly lower in neuroblastoma samples with *MYCN* amplification (median values: 2.42 for p85α and 4.63 for p110δ) than without amplification (median values: 0.81 for p85α and 0.75 for p110δ) (Exact Wilcoxon rank-sum test, $p=0.0339$ for p85α; $p=0.0339$ for p110δ). In contrast, no correlation was found between p85α and p110δ expression and tumor stage, 1p status or progression. Thus, p85α and p110δ expression was found to be increased in neuroblastoma samples from children under the age of one and with no *MYCN* amplification.

To investigate if *PIK3CD* mRNA levels are predictive of protein expression levels, Western blot analysis was performed on twelve of the patient samples. In 10/12 samples, mRNA levels correlated with protein expression (Fig 1A, right panel).

Reanalysis of cDNA microarray data of an independent panel of 68 primary neuroblastoma samples also revealed striking variations in the expression levels of the class I_A PI3K regulatory subunit p85α and the catalytic subunit p110δ. Expression of the p85α and p110δ subunits was significantly higher in samples from children under the age of one

compared to patients older than one year of age (Fig. 1B), confirming the results of the qRT-PCR analysis (Fig. 1A).

We next investigated the expression of PI3K isoforms in a set of 8 representative neuroblastoma cell lines to validate the findings obtained in tumor samples. In this panel of cell lines, p85α was over-expressed in 5/8 samples, while the catalytic isoforms p110α, p110β and p110δ showed increased expression in 4/8, 6/8 and 1/8 cell lines, respectively (Fig 1C, left panel). In line with the findings in patient samples, cell lines harbouring *MYC* amplification showed decreased levels of *PIK3CD* mRNA when compared to adrenal tissue (Fig 1C, left panel). As with the patient samples, mRNA levels were largely found to be predictive of protein levels (Fig 1C, right panel).

Insert Figure 1

The expression of PI3K isoforms was next investigated at the protein level in a panel of 8 neuroblastoma cell lines. All neuroblastoma cell lines included in this study expressed the p85α, p110α and p110β isoforms, while the expression of p110δ was more variable, being highest in SH-SY5Y cells (Fig. 2A). Together, these data show that the expression levels of PI3K isoforms are altered in neuroblastoma samples and cell lines at the mRNA and/or protein level, and that the expression of the catalytic p110δ isoform is increased in neuroblastoma.

Insert Figure 2

Activation of PI3K/Akt signalling by polypeptide growth factors in neuroblastoma cell lines. Class I_A PI3Ks transduce signals from activated RTKs to downstream effectors, the most important of which is the Ser/Thr protein kinase Akt (12, 13). The activation of Akt by polypeptide growth factors was investigated in SH-SY5Y and LA-N-1 cells. Epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) most potently activated Akt in SH-SY5Y cells, while platelet-derived growth factor (PDGF) and IGF-1 induced the strongest response in LA-N-1 cells (Fig. 2B).

EGF and IGF-1 stimulated growth of SH-SY5Y (2 and 2.5-fold) and LA-N-1 (2 and 2.2-fold) cells, while PDGF was less potent at promoting a growth response (Fig. 2C). The pharmacological PI3K inhibitor LY294002 reduced basal growth of SH-SY5Y (66.5%

inhibition) and LA-N-1 (42.8% inhibition) cells. Treatment with the PI3K inhibitor also reduced EGF- and IGF-1-stimulated growth in SH-SY5Y (44.1% and 27.6% inhibition respectively) and LA-N-1 cells (Fig. 2C). However, LY294002 as a single agent did not significantly affect the extent of growth factor-stimulated cell growth in either SH-SY5Y or LA-N-1 cells (Fig. 2C). Recently, a similar observation was reported in neuroblastoma cell lines, where the combination of both LY294002 and the mTOR inhibitor rapamycin was required to inhibit IGF-1 induced proliferation (9). Thus, various growth factors, including EGF and IGF-1, stimulate growth of neuroblastoma cells via the PI3K/Akt pathway.

Impact of shRNA-mediated down-regulation of p110α and p110δ on neuroblastoma cell proliferation and apoptosis. To gain insight into the individual functions of class I_A PI3K isoforms in neuroblastoma cell responses, SH-SY5Y and LA-N-1 cells were stably transduced with short hairpin RNA (shRNA) constructs targeting p110α (hereinafter $p110\alpha^{low}$) or p110δ (hereinafter $p110\delta^{low}$). Specific down-regulation of target gene expression was verified by Western blot analysis (Fig. 3A). The growth of SH-SY5Y $p110\delta^{low}$ cells was significantly reduced (53.1% and 44.1% inhibition) when the cells were cultivated in medium containing low (1%) serum, but not high (10%) serum (Fig. 3B). This effect was less pronounced in LA-N-1 cells (Fig. 3B), correlating with the differential expression of p110δ in these cell lines. While an effect of p110α down-regulation was also observed in SH-SY5Y cells (low serum: no inhibition or 21.3% inhibition; high serum: no inhibition or 21.2% inhibition), the effect was stronger in LA-N-1 cells (low serum: 22.1% and 66.4% inhibition; high serum: 11.7% and 43.4%,inhibition).

To investigate whether PI3K targeting promoted apoptosis in neuroblastoma cells, caspase activity and cell cycle distribution were analysed in SH-SY5Y $p110\alpha^{low}$ or $p110\delta^{low}$ cells. A significant (2.2-fold) increase in caspase-3/7 activity was observed in SH-SY5Y $p110\delta^{low}$ cells (Fig. 3C). In contrast, a comparable response was not observed in SH-SY5Y $p110\alpha^{low}$ cells (Fig. 3C). Analysis of cell cycle distribution revealed a decrease in the S-phase in SH-SY5Y $p110\alpha^{low}$ and $p110\delta^{low}$ cells, both in low serum (61.8% decrease in both cases) and high serum (71.4% and 59.2% decrease). The increase in cell death under low serum conditions was higher in SH-SY5Y $p110\delta^{low}$ cells (319.4% increase) than in SH-SY5Y $p110\alpha^{low}$ cells (64.5% increase), confirming the results of the caspase assay (Fig. 3C). Together, these results demonstrate that the p110δ isoform plays a major role in SH-SY5Y cell growth and survival under suboptimal culture conditions. In contrast, a comparable role

was not apparent for p110α in these cells, although this isoform appeared to play a more important function in LA-N-1 cells, which express only very low levels of p110δ.

In view of the increased apoptosis observed in SH-SY5Y p110δ^{low} cells, we analysed the expression of pro- and anti-apoptotic Bcl-2 family proteins, which are key regulators of apoptosis. The levels of Bcl-2 and Bcl-X_L were elevated in SH-SY5Y cells grown in high serum, as compared to low serum conditions (Fig. 3D and 5B). When compared to control cells, SH-SY5Y p110δ^{low} cells displayed reduced expression of the anti-apoptotic proteins Bcl-2 and Bcl-X_L (Fig. 3D and 5B). In contrast, the levels of Bax were unaffected (Fig. 3D). Thus, p110δ appears to contribute to the control of the expression of anti-apoptotic Bcl-2 family proteins, which may impact on its function in neuroblastoma cell survival.

Insert Figure 3

Impact of shRNA-mediated down-regulation of p110α and p110δ on neuroblastoma cell responses to growth factors. We next investigated the involvement of class I_A PI3K isoforms in polypeptide growth factor signalling in neuroblastoma cells. The ability of EGF and IGF-1 to stimulate the growth of SH-SY5Y cells was significantly reduced in both SH-SY5Y p110α^{low} and p110δ^{low} cells (EGF: 6.4% and 40.3%, 23.4% and 16.2%, respectively; IGF-1: 27.5% and 41.5%, 42.1% and 49.1%, respectively) (Fig. 4A). In contrast, the ability of EGF and IGF-1 to stimulate the growth of LA-N-1 p110α^{low} and p110δ^{low} cells was not significantly impaired (Fig. 4A).

The impact of the shRNA constructs targeting p110α and p110δ on the activation of early signalling mediators of RTKs was then investigated in SH-SY5Y and LA-N-1 cells. Activation of Akt by EGF and IGF-1 was unaltered in SH-SY5Y and LA-N-1 cells (Fig. 4B and Supplemental Fig. 2). In contrast, activation of the mTOR/S6K pathway by EGF and IGF-1 was impaired in both SH-SY5Y p110α^{low} and p110δ^{low} cells, as assessed by phosphorylation of the S6 protein (Fig. 4B). While the impact of p110α down-regulation on signal transduction was expected, the observation that decreased p110δ expression leads to a comparable effect on its own was surprising. Activation of Erk1/2 by EGF and IGF-1 was unaffected by the PI3K shRNAs (data not shown). Thus, p110δ plays an important role in activation of the mTOR/S6K pathway in SH-SY5Y cells, which correlates with its involvement in neuroblastoma cell growth. However, activation of Akt by growth factors is insensitive to down-regulation of a single PI3K isoform in these cells. This is not unexpected,

as previous studies have reported that PI3K isoforms from other classes contribute to Akt activation (24). In both LA-N-1 p110α^{low} and p110δ^{low} cells, basal activation of Akt and S6K was reduced (Supplemental Fig. 2). However, in LA-N-1 cells, phosphorylation of the S6 protein was hardly affected by down-regulation of p110δ (Supplemental Fig. 2). While decreased p110α expression led to a stronger inhibition of downstream signalling, no apparent effect on the proliferative response was observed, suggesting that other pathways may compensate for reduced signal transduction in this cell line (Fig. 4A and Supplemental Fig. 2).

Insert Figure 4

Constitutive activation of the S6K pathway abrogates the effect of p110δ shRNA on SH-SY5Y cell growth. Our previous data had shown a marked effect of p110δ down-regulation by shRNA on activation of the mTOR/S6K pathway in SH-SY5Y cells, contributing to cell growth. To confirm this model, SH-SY5Y p110δ^{low} cells were transiently transfected with an activated mutant of S6K1. Activated S6K promoted growth of SH-SY5Y cells and was able partially rescue the growth of SH-SY5Y p110δ^{low} cells (Fig. 5A). However, the induction of apoptosis induced by the p110δ shRNA was only marginally impaired by S6K transfection, as assessed by cleavage of poly (ADP-ribose) polymerase (PARP) (Fig. 5B). Moreover, transient transfection of activated S6K did not restore the levels of Bcl-2 and Bcl-X_L in SH-SY5Y p110δ^{low} cells (Fig. 5B). Together, these results demonstrate that the PI3K p110δ isoform controls neuroblastoma cell growth via the S6K pathway. In addition, it also appears to play a role in controlling the levels of Bcl-2 family proteins, which appears not to involve S6K or Akt.

Insert Figure 5

Rapamycin mimics the effects of p110δ down-regulation. The data presented above highlighted the importance of signalling via the mTOR/S6K pathway in the regulation of neuroblastoma cell survival and the ability to respond to growth factors. To confirm these findings, SH-SY5Y and LA-N-1 cells were treated with rapamycin, an mTOR inhibitor, and cellular responses were investigated. As expected, phosphorylation of the S6 protein was abrogated in the presence of rapamycin, both in low (1%) and high (10%) serum conditions (Fig. 6A). Treatment of SH-SY5Y and LA-N-1 cells with rapamycin led to a dose-dependent

inhibition of basal cell proliferation (SH-SY5Y: 29.2% (20ng/ml) and 56.6% (100ng/ml) inhibition; LA-N-1: 37.3% (20ng/ml) and 61.8% (100ng/ml) inhibition) (Fig. 6B). Co-treatment of cells with rapamycin attenuated the proliferative response to EGF (SH-SY5Y: 34.7% (20ng/ml) and 55.8% (100ng/ml) inhibition; LA-N-1: 32.5% (20ng/ml) and 44.4% (100ng/ml) inhibition) and IGF-1 (SH-SY5Y: 32.5% (20ng/ml) and 53% (100ng/ml) inhibition; LA-N-1: 27.7% (20ng/ml) and 42.1% (100ng/ml) inhibition) (Fig. 6B). Furthermore, phosphorylation of the S6 protein was found to be absent upon stimulation with EGF or IGF-1 in cells pre-treated with rapamycin (Fig. 6C). These findings emphasise the importance of intact mTOR/S6K signalling in neuroblastoma cell survival.

Insert Figure 6

Discussion

In recent years, PI3K has emerged as a central controller of the cellular responses to a variety of growth factors and PI3K signalling is frequently deregulated by diverse mechanisms in human cancer (14, 16). PI3K has also been shown to play an important role in neuroblastoma biology. Therefore, targeting PI3K may represent an attractive approach to inhibit neuroblastoma proliferation *in vivo* (11, 17, 18, 25). However, the mechanisms contributing to the activation of PI3K signalling in neuroblastoma are still unclear, since loss of *PTEN* or *PIK3CA* mutations are uncommon in this paediatric malignancy (26-28). We show here for the first time that the class I_A PI3K isoform p110δ plays a crucial role in neuroblastoma cell growth and survival and that the expression of p110δ is increased at the mRNA and protein level in a subset of primary neuroblastoma samples and cell lines. In addition, our data reveal a selective expression of p85α/p110δ in neuroblastoma samples from children under the age of one. Our results also reveal a negative correlation between *PIK3CD* gene expression and *MYCN* amplification. Expression of *PIK3CD* was recently shown to be significantly lower in neuroblastoma samples with LOH at 1p36 (29). A recent study also described a correlation between decreased *PIK3CD* expression, 1p deletion and poor clinical outcome (30). LOH at 1p36 was associated with amplification of the *MYCN* oncogene (31). Reduced expression of *PIK3CD* in neuroblastoma samples may thus correlate with poor prognosis, due to its association with age at diagnosis (>1 year), LOH at 1p36 and/or *MYCN* amplification, since these events are prognostic markers of poor outcome in neuroblastoma (1, 2, 31, 32). N-Myc was shown to be able to replace IGF/PI3K signalling in medulloblastoma formation (33). Moreover, c-Myc expression had a negative impact on the activation of PI3K/Akt signalling (34). Thus, it is conceivable that *MYCN*-amplified neuroblastoma display decreased *PIK3CD* expression, either because of: (i) LOH at 1p36 (29); (ii) a direct negative effect of *MYCN* amplification on *PIK3CD* expression; or (iii) because they are less dependent on p85α/p110δ signalling. Collectively, these data indicate that the class I_A p110δ isoform may play a role in the development of neuroblastoma in very young patients (<1 year of age), or in a subset of tumours which do not harbour LOH at 1p36 and/or *MYCN* amplification.

The expression of p110δ was previously shown to be predominantly restricted to leukocytes in normal tissues (35). However, different human cancer cell lines were also shown to express this PI3K isoform, indicating that aberrant expression of p110δ in tumours may contribute to the malignant properties of the cancer cells (24, 36). In support of this notion, the

ability of p110δ to induce transformation of chicken fibroblasts was recently demonstrated (37). In breast cancer cell lines, a selective function of p110δ in cell migration was also described (36). In acute myeloid leukemia, recent reports have shown that p110δ plays a major role in activation of Akt by RTKs (FLT3), cell proliferation and chemoresistance (38, 39). We show here that the class I_A PI3K isoforms p110α and p110δ do not have overlapping functions in neuroblastoma cell responses. Indeed, in SH-SY5Y cells which display increased *PIK3CA* and *PIK3CD* expression, p110δ appeared to play a major role in controlling neuroblastoma cell growth and survival under limiting growth conditions. In contrast, in LA-N-1 cells with only very low p110δ expression, down-regulation of p110α by shRNA impaired cell growth and Akt activation. The impact of p110δ down-regulation by shRNA on SH-SY5Y growth correlated with an impairment of the activation of the mTOR/S6K pathway. Surprisingly, Akt activation by EGF and IGF-1 was unaffected by p110δ down-regulation, under conditions where the mTOR/S6K pathway was inhibited. Thus, activation of Akt and mTOR/S6K have different sensitivities to class I_A PI3K down-regulation. It is conceivable that other PI3K isoforms may compensate for p110α or p110δ in growth factor-stimulated Akt activation (24). Transfection of an activated mutant of S6K was sufficient to partially rescue the growth defect of SH-SY5Y p110δ^{low} cells under low serum conditions. However, p110δ appeared to have an additional function in maintaining the levels of anti-apoptotic Bcl-2 family proteins in neuroblastoma cells, which did not involve S6K or Akt. The observation that the expression levels of anti-apoptotic Bcl-2 family proteins were higher in SH-SY5Y under high serum conditions, than in low serum, indicates that growth factors contribute to the regulation of the levels of Bcl-2 family proteins in these cells. Previous reports have documented a role for IGF-1, which is present in the FCS, in maintaining Bcl-2 (40) and Bcl-X_L (41) expression levels. In view of the data presented here showing a role for p110δ in IGF-1 signalling in neuroblastoma cells, it can be postulated that IGF-1R/p110δ contributes to regulation of the expression of Bcl-2 family proteins. Since Akt and S6K were not involved in this pathway, the simplest explanation for these observations is that mTOR-mediated phosphorylation of 4E-BP1 controls translational activation of the expression of Bcl-2 and/or Bcl-X_L in neuroblastoma cells, which is supported by previous findings in other systems (42). Down-regulation of p110α by shRNA had a less pronounced impact on the growth of SH-SY5Y cells under low serum conditions, although activation of S6K was impaired. In view of the observation that p110α down-regulation did not induce apoptosis in SH-SY5Y cells, in

contrast to p110δ, it can be speculated that Bcl-2 family proteins are selectively controlled by the p110δ isoform.

Together, our results demonstrate that p110δ contributes to neuroblastoma cell growth and survival by regulating the activation of the mTOR/S6K pathway and the expression levels of anti-apoptotic Bcl-2 family proteins.

Acknowledgements

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References

1. Schwab M, Westermann F, Hero B, Berthold F. Neuroblastoma: biology and molecular and chromosomal pathology. Lancet Oncol 2003;4: 472-80.
2. Brodeur GM. Neuroblastoma: biological insights into a clinical enigma. Nat Rev Cancer 2003;3: 203-16.
3. Matthay KK, Villablanca JG, Seeger RC, *et al.* Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group. The New England journal of medicine 1999;341: 1165-73.
4. Meyer GE, Shelden E, Kim B, Feldman EL. Insulin-like growth factor I stimulates motility in human neuroblastoma cells. Oncogene 2001;20: 7542-50.
5. Singleton JR, Randolph AE, Feldman EL. Insulin-like growth factor I receptor prevents apoptosis and enhances neuroblastoma tumorigenesis. Cancer research 1996;56: 4522-9.
6. Leventhal PS, Randolph AE, Vesbit TE, Schenone A, Windebank A, Feldman EL. Insulin-like growth factor-II as a paracrine growth factor in human neuroblastoma cells. Exp Cell Res 1995;221: 179-86.
7. El-Badry OM, Helman LJ, Chatten J, Steinberg SM, Evans AE, Israel MA. Insulin-like growth factor II-mediated proliferation of human neuroblastoma. J Clin Invest 1991;87: 648-57.
8. Cianfarani S, Rossi P. Neuroblastoma and insulin-like growth factor system. New insights and clinical perspectives. Eur J Pediatr 1997;156: 256-61.
9. Guerreiro AS, Boller D, Shalaby T, Grotzer MA, Arcaro A. Protein kinase B modulates the sensitivity of human neuroblastoma cells to insulin-like growth factor receptor inhibition. International journal of cancer 2006;119: 2527-38.
10. Beppu K, Jaboine J, Merchant MS, Mackall CL, Thiele CJ. Effect of imatinib mesylate on neuroblastoma tumorigenesis and vascular endothelial growth factor expression. J Natl Cancer Inst 2004;96: 46-55.
11. Ho R, Eggert A, Hishiki T, *et al.* Resistance to chemotherapy mediated by TrkB in neuroblastomas. Cancer research 2002;62: 6462-6.
12. Vanhaesebroeck B, Leevers SJ, Ahmadi K, *et al.* Synthesis and function of 3-phosphorylated inositol lipids. Annu Rev Biochem 2001;70: 535-602.
13. Kozma SC, Thomas G. Regulation of cell size in growth, development and human disease: PI3K, PKB and S6K. Bioessays 2002;24: 65-71.
14. Katso R, Okkenhaug K, Ahmadi K, White S, Timms J, Waterfield MD. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. Annu Rev Cell Dev Biol 2001;17: 615-75.
15. Cantley LC, Neel BG. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proceedings of the National Academy of Sciences of the United States of America 1999;96: 4240-5.
16. Weir B, Zhao X, Meyerson M. Somatic alterations in the human cancer genome. Cancer Cell 2004;6: 433-8.
17. Jaboin J, Kim CJ, Kaplan DR, Thiele CJ. Brain-derived neurotrophic factor activation of TrkB protects neuroblastoma cells from chemotherapy-induced apoptosis via phosphatidylinositol 3'-kinase pathway. Cancer research 2002;62: 6756-63.
18. Kim B, van Golen CM, Feldman EL. Insulin-like growth factor-I signaling in human neuroblastoma cells. Oncogene 2004;23: 130-41.

19. Beppu K, Nakamura K, Linehan WM, Rapisarda A, Thiele CJ. Topotecan blocks hypoxia-inducible factor-1alpha and vascular endothelial growth factor expression induced by insulin-like growth factor-I in neuroblastoma cells. Cancer research 2005;65: 4775-81.
20. Ho R, Minturn JE, Hishiki T, *et al.* Proliferation of human neuroblastomas mediated by the epidermal growth factor receptor. Cancer research 2005;65: 9868-75.
21. Schramm A, Schulte JH, Klein-Hitpass L, *et al.* Prediction of clinical outcome and biological characterization of neuroblastoma by expression profiling. Oncogene 2005;24: 7902-12.
22. Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods (San Diego, Calif 2001;25: 386-401.
23. Paddison PJ, Silva JM, Conklin DS, *et al.* A resource for large-scale RNA-interference-based screens in mammals. Nature 2004;428: 427-31.
24. Arcaro A, Khanzada UK, Vanhaesebroeck B, Tetley TD, Waterfield MD, Seckl MJ. Two distinct phosphoinositide 3-kinases mediate polypeptide growth factor-stimulated PKB activation. The EMBO journal 2002;21: 5097-108.
25. Chesler L, Schlieve C, Goldenberg DD, *et al.* Inhibition of Phosphatidylinositol 3-Kinase Destabilizes Mycn Protein and Blocks Malignant Progression in Neuroblastoma. Cancer research 2006;66: 8139-46.
26. Moritake H, Horii Y, Kuroda H, Sugimoto T. Analysis of PTEN/MMAC1 alteration in neuroblastoma. Cancer genetics and cytogenetics 2001;125: 151-5.
27. Munoz J, Lazcoz P, Inda MM, *et al.* Homozygous deletion and expression of PTEN and DMBT1 in human primary neuroblastoma and cell lines. International journal of cancer 2004;109: 673-9.
28. Dam V, Morgan BT, Mazanek P, Hogarty MD. Mutations in PIK3CA are infrequent in neuroblastoma. BMC Cancer 2006;6: 177.
29. Wang Q, Diskin S, Rappaport E, *et al.* Integrative genomics identifies distinct molecular classes of neuroblastoma and shows that multiple genes are targeted by regional alterations in DNA copy number. Cancer research 2006;66: 6050-62.
30. Fransson S, Martinsson T, Ejeskar K. Neuroblastoma tumors with favorable and unfavorable outcomes: Significant differences in mRNA expression of genes mapped at 1p36.2. Genes, chromosomes & cancer 2006.
31. Attiyeh EF, London WB, Mosse YP, *et al.* Chromosome 1p and 11q deletions and outcome in neuroblastoma. The New England journal of medicine 2005;353: 2243-53.
32. Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM. Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. Science (New York, NY 1984;224: 1121-4.
33. Browd SR, Kenney AM, Gottfried ON, *et al.* N-myc can substitute for insulin-like growth factor signaling in a mouse model of sonic hedgehog-induced medulloblastoma. Cancer research 2006;66: 2666-72.
34. Bellmann K, Martel J, Poirier DJ, Labrie MM, Landry J. Downregulation of the PI3K/Akt survival pathway in cells with deregulated expression of c-Myc. Apoptosis 2006;11: 1311-9.
35. Vanhaesebroeck B, Welham MJ, Kotani K, *et al.* P110delta, a novel phosphoinositide 3-kinase in leukocytes. Proceedings of the National Academy of Sciences of the United States of America 1997;94: 4330-5.
36. Sawyer C, Sturge J, Bennett DC, *et al.* Regulation of breast cancer cell chemotaxis by the phosphoinositide 3-kinase p110delta. Cancer research 2003;63: 1667-75.
37. Kang S, Denley A, Vanhaesebroeck B, Vogt PK. Oncogenic transformation induced by the p110beta, -gamma, and -delta isoforms of class I phosphoinositide 3-kinase.

Proceedings of the National Academy of Sciences of the United States of America 2006;103: 1289-94.

38. Sujobert P, Bardet V, Cornillet-Lefebvre P, *et al.* Essential role for the p110delta isoform in phosphoinositide 3-kinase activation and cell proliferation in acute myeloid leukemia. Blood 2005;106: 1063-6.

39. Billottet C, Grandage VL, Gale RE, *et al.* A selective inhibitor of the p110delta isoform of PI 3-kinase inhibits AML cell proliferation and survival and increases the cytotoxic effects of VP16. Oncogene 2006;25: 6648-59.

40. Minshall C, Arkins S, Straza J, *et al.* IL-4 and insulin-like growth factor-I inhibit the decline in Bcl-2 and promote the survival of IL-3-deprived myeloid progenitors. J Immunol 1997;159: 1225-32.

41. Parrizas M, Saltiel AR, LeRoith D. Insulin-like growth factor 1 inhibits apoptosis using the phosphatidylinositol 3'-kinase and mitogen-activated protein kinase pathways. The Journal of biological chemistry 1997;272: 154-61.

42. Wu LX, La Rose J, Chen L, *et al.* CD28 regulates the translation of Bcl-xL via the phosphatidylinositol 3-kinase/mammalian target of rapamycin pathway. J Immunol 2005;174: 180-94.

Figure Legends

Fig. 1. Expression of class I_A PI3K genes in neuroblastoma patient samples and cell lines. *A,* TaqMan analysis of class I_A PI3K mRNA levels in neuroblastoma patient samples (n=19) and human adrenal gland (AG). *PIK3R1*: black bars, *PIK3CA*: light grey bars, *PIK3CB*: white bars, *PIK3CD*: dark grey bars (*left panel*). Western blot of p110δ expression in patient samples (n=12) and AG compared to TaqMan data (*right panel*) *B,* Box plot visualising p85α and p110δ expression in an additional, independent group of 68 human neuroblastoma samples. *D*, Analysis of class I_A PI3K mRNA levels in human neuroblastoma cell lines (n=8) and AG. Colour code according to *A* (*left panel*). Western blot of p110δ expression in cell lines (n=8) and AG compared to TaqMan data (*right panel*).

Fig. 2. PI3K class I_A isoform expression and growth factor-induced pathway activation in human neuroblastoma cell lines. *A,* Expression pattern of PI3K isoforms, Akt and the PI3K antagonist phosphatase and tensin homolog (PTEN) in human neuroblastoma cell lines. *B,* Growth factor-induced pathway activation in SH-SY5Y and LA-N-1 cells. *C,* Effect of LY294002 on growth factor-stimulated proliferation.

Fig. 3. Effect of class I_A PI3K isoform down-regulation on cell proliferation and cell death in human neuroblastoma cells. *A,* Western blot analysis of protein down-regulation in SH-SY5Y and LA-N-1 cells stably transfected with shRNA against p110α (PIK3CA) or p110δ (PIK3CD) or the control vector (pRS). *B,* Basal proliferation of SH-SY5Y or LA-N-1 cells in medium containing low (1%) or high (10%). *C*, Basal caspase-3/7 activity of SH-SY5Y clones in medium containing low (1%) or high (10%) serum (*left panel*). Cell cycle distribution of SH-SY5Y clones in medium containing low (1%) serum. Dead cells: black fraction; G2-phase: dark grey fraction; S-phase: white fraction; G0/G1-phase: light grey fraction (*right panel*) *D*, Western blot analysis of the expression levels of proteins involved in apoptosis in SH-SY5Y cells stably transfected with shRNA against p110δ (PIK3CD) or the control vector (pRS) under low (1%) and high (10%) serum conditions.

Fig. 4. Proliferative response and pathway activation upon growth factor stimulation in cells expressing decreased levels of p110α or p110δ. *A*, Growth factor-induced proliferation of SH-SY5Y and LA-N-1 cells stably transfected with shRNA against p110α (light grey bars)

or p110δ (dark grey bars) or the control vector pRS (black bars). *B*, Growth factor-induced activation of the PI3K pathway in SH-SY5Y p110α^{low} (PIK3CA) or p110δ^{low} (PIK3CD) cells compared to control cells (pRS).

Fig. 5. Constitutively activated S6 kinase can partially rescue SH-SY5Y cells from cell death induced by p110δ down-regulation. *A*, Proliferative response of SH-SY5Y p110δ^{low} cells (PIK3CD) transiently transfected with constitutively activated S6 kinase 1 (S6K AK1) or the control vector (pcDNA3). *B,* Western blot analysis of the expression levels of proteins involved in apoptosis (Bcl2, Bcl-X_L, Bax) and PARP cleavage.

Fig. 6. Rapamycin mimics the effect of p110δ down-regulation. *A,* Western blot analysis of S6 protein phosphorylation upon treatment of SH-SY5Y and LA-N-1 cells with rapamycin. *B*, Proliferation of SH-SY5Y and LA-N-1 cells in response to EGF and IGF-1 upon co-treatment with rapamycin (ng/ml where indicated). *C*, Western blot analysis of S6 protein phosphorylation in response to stimulation with EGF or IGF-1 upon pre-treatment with rapamycin.

Supplemental Fig. 1. Characteristics of human neuroblastoma patient samples as determined by histological assessment of the tumor specimen obtained at surgery. nd: not determined

Supplemental Fig. 2. Growth factor-induced activation of the PI3K pathway in LA-N-1 p110α^{low} (PIK3CA) or p110δ^{low} (PIK3CD) cells compared to control cells (pRS).

Figure 1

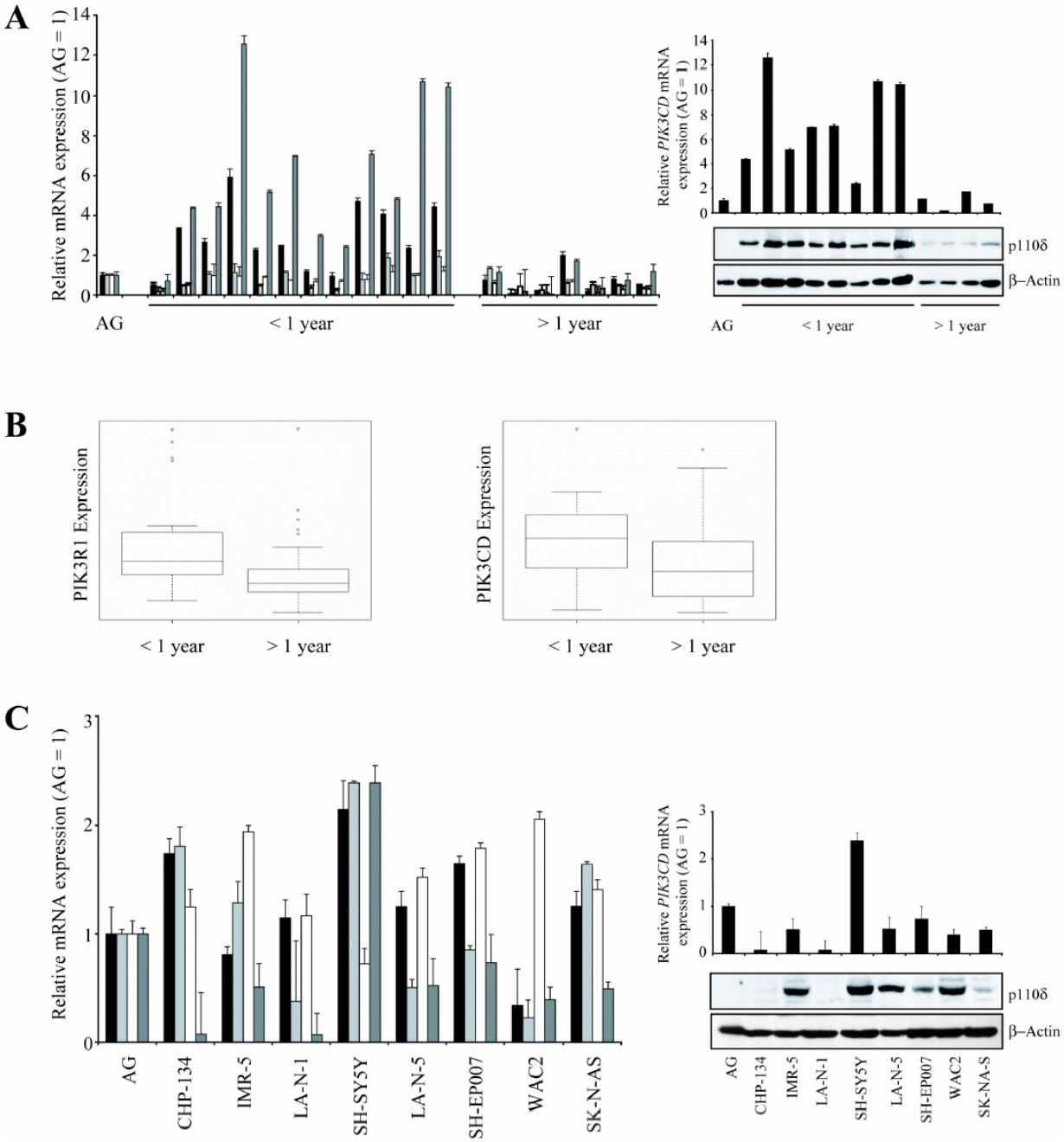

A
Relative mRNA expression (AG = 1)
AG
< 1 year
> 1 year
Relative PIK3CD mRNA expression (AG = 1)
p110δ
β–Actin
B
PIK3R1 Expression
PIK3CD Expression
C
CHP-134
IMR-5
LA-N-1
SH-SY5Y
LA-N-5
SH-EP007
WAC2
SK-N-AS
SK-NA-S

Figure 2

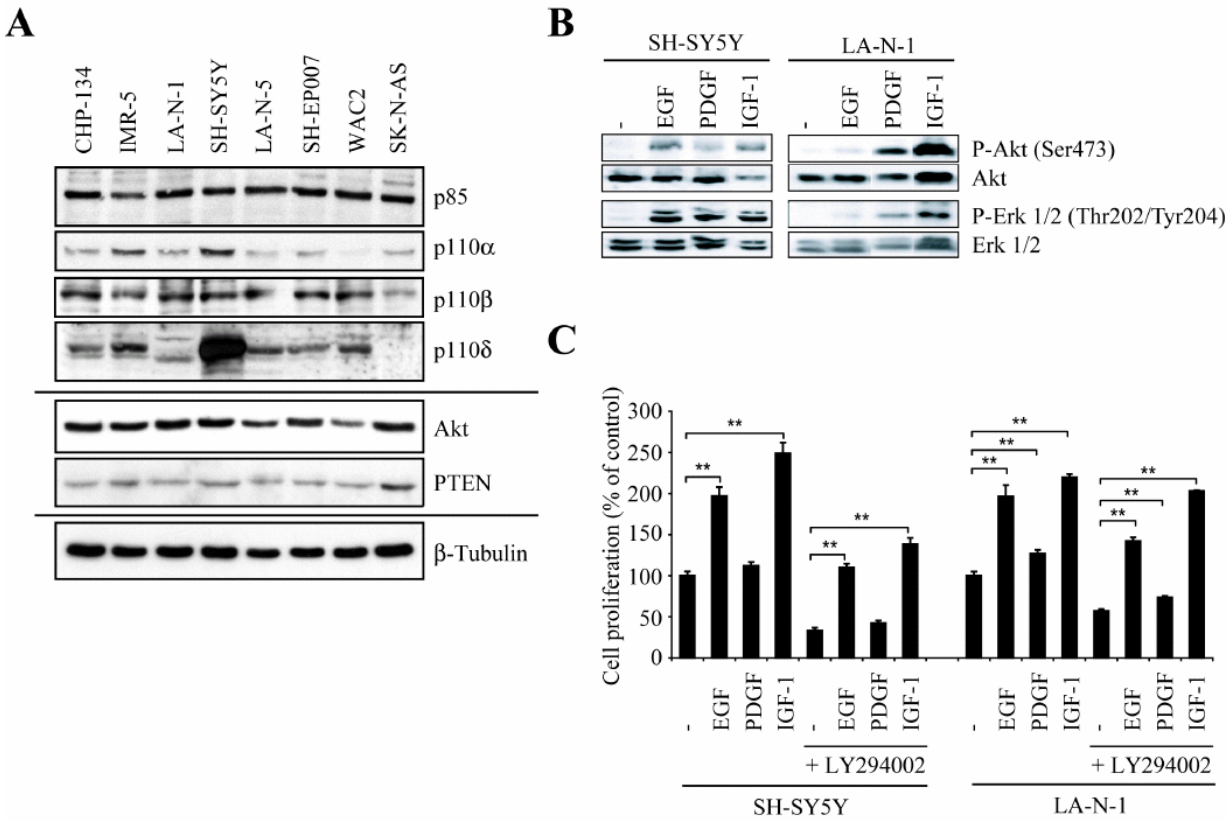

A
CHP-134
IMR-5
LA-N-1
SH-SY5Y
LA-N-5
SH-EP007
WAC2
SK-N-AS
p85
p110α
p110β
p110δ
Akt
PTEN
β-Tubulin
B
SH-SY5Y
LA-N-1
-
EGF
PDGF
IGF-1
P-Akt (Ser473)
Akt
P-Erk 1/2 (Thr202/Tyr204)
Erk 1/2
C
Cell proliferation (% of control)
300
250
200
150
100
50
0
**
+ LY294002
SH-SY5Y
LA-N-1

Figure 3

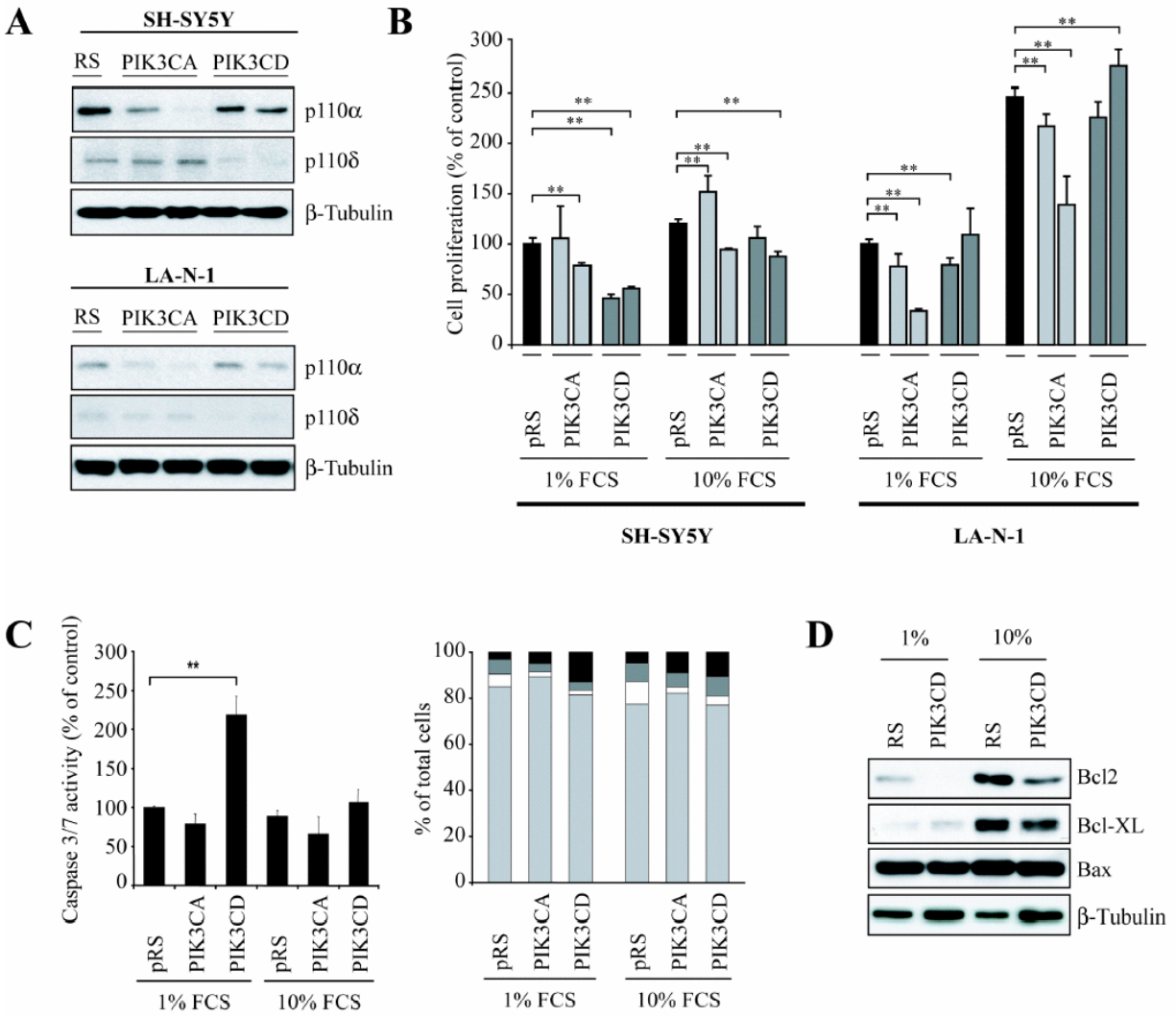

A
SH-SY5Y
RS
PIK3CA
PIK3CD
p110α
p110δ
β-Tubulin
LA-N-1
RS
PIK3CA
PIK3CD
p110α
p110δ
β-Tubulin
B
Cell proliferation (% of control)
300
250
200
150
100
50
0
**
pRS
PIK3CA
PIK3CD
1% FCS
10% FCS
SH-SY5Y
LA-N-1
C
Caspase 3/7 activity (% of control)
300
250
200
150
100
50
0
**
pRS
PIK3CA
PIK3CD
1% FCS
10% FCS
% of total cells
100
80
60
40
20
0
D
1%
10%
RS
PIK3CD
Bcl2
Bcl-XL
Bax
β-Tubulin

Figure 4

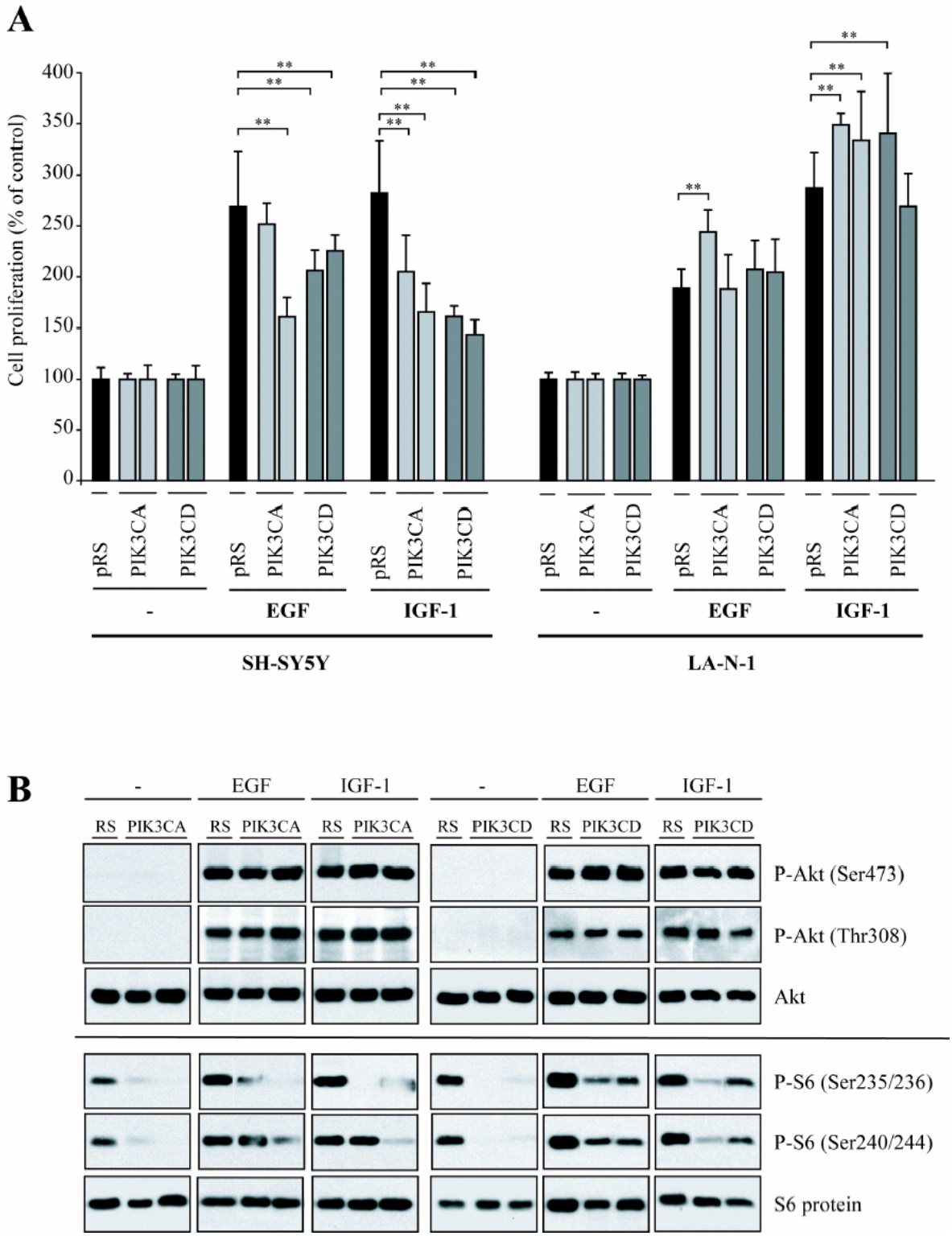

A
Cell proliferation (% of control)
400
350
300
250
200
150
100
50
0
**
pRS
PIK3CA
PIK3CD
-
EGF
IGF-1
SH-SY5Y
LA-N-1
B
RS PIK3CA
RS PIK3CD
P-Akt (Ser473)
P-Akt (Thr308)
Akt
P-S6 (Ser235/236)
P-S6 (Ser240/244)
S6 protein

Figure 5

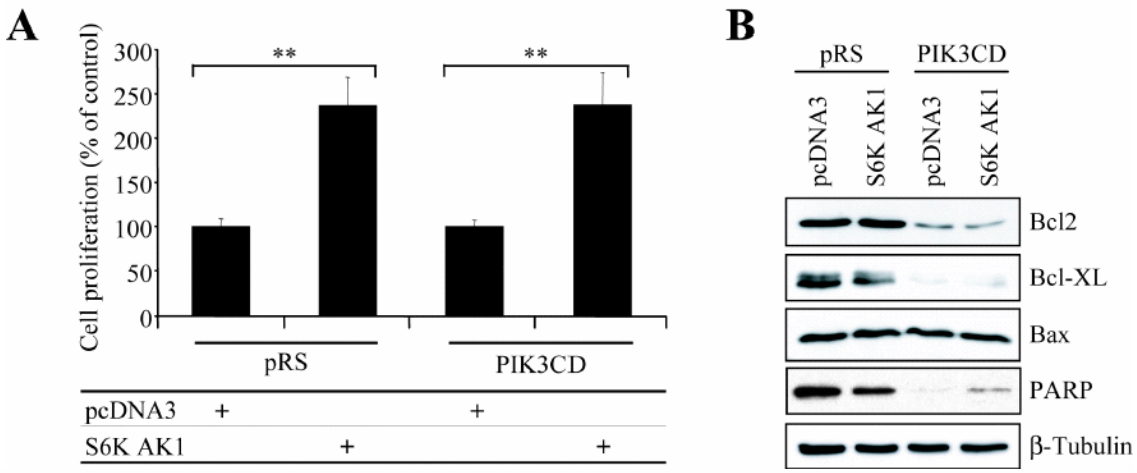
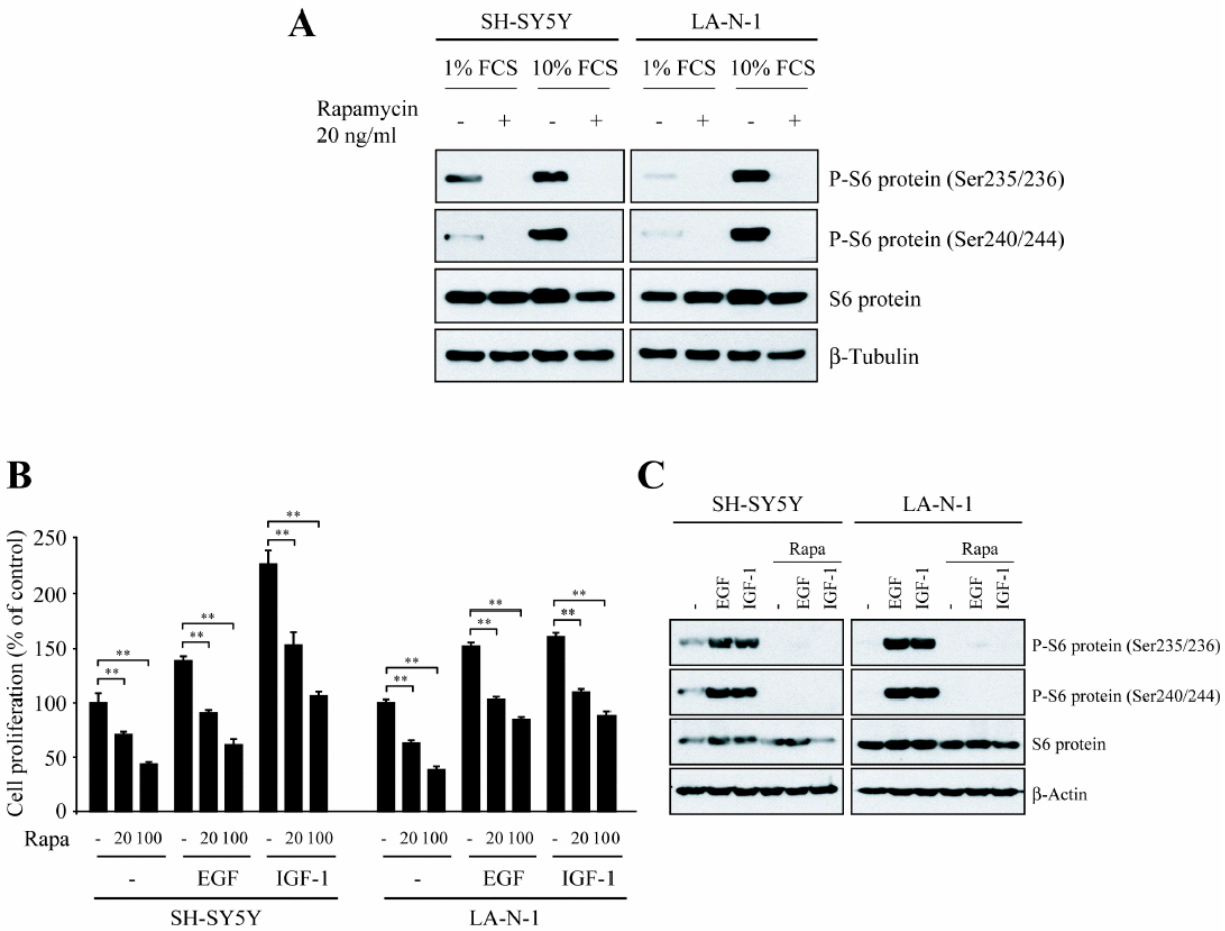


Figure 6



4. CONCLUSIONS AND PERSPECTIVES

A better understanding of the mechanisms underlying tumor formation has resulted in a new era of cancer treatment strategies using highly-specific inhibitors for gene targeted therapies. Extensive research aimed at identifying promising new molecular targets has placed RTK signaling into the front ranks. Several studies have proven a high efficacy of targeting the RTK system in cancer treatment and the use of novel small-molecule inhibitors lead to promising results and greatly reduced incidence rates of adverse side effects. Moreover, a trend towards combinatorial therapies, in which multiple signaling pathways are being targeted at the same time, is arising. Over the last years, great progress has been made in understanding the pathogenesis of AML revealing a number of promising targets, such as FLT3 and c-Kit. Despite the fact that clinical trials have corroborated the feasibility of using these newly developed agents in AML and have demonstrated acceptable tolerabilities in patients, they are not yet used as a standard treatment. Moreover, due to the heterogeneity of the disease, the inhibitors currently in clinical trials have only shown satisfactory results in certain subgroups of patients. Therefore, a number of other molecular targets remain yet to be discovered and this study was aimed at extending the current understanding of RTK signaling in human cancers, AML in particular, and the field of novel useful approaches.

The studies presented in this thesis on the IGF-IR/PI3K signaling system in AML blasts and cell lines highlighted the significant contribution of this network to cancer cell survival. We could show that IGF-I promotes growth and survival of AML blasts and that it activates intracellular signaling mediators including Akt and ERK. Furthermore, high expression levels of the IGF-IR were found in a panel of primary AML blasts and cell lines. Besides, we discovered that IGF-I was consistently secreted by the AML cells implying the presence of an autocrine signaling loop involving IGF-I and the IGF-IR. Previous studies had already described expression of the IGF-IR in human leukemias and over-expression of the IGF-IR was stated to harbinger transformation from MDS to AML as the IGF-IR appeared to have higher to lower expression rate in turn from AML to MDS to normal controls (Qi et al., 2006; Zumkeller & Burdach, 1999). Moreover, the expression of the IGF-IR negatively correlated with apoptosis suggesting a protective role for this receptor (Qi et al., 2006). Our finding of an autocrine IGF-I production and the involvement of the IGF-I/IGF-IR signaling system in AML cell growth and proliferation could be a more

relevant explanation for that survival advantage. Indeed we could show that blockage of the IGF-IR by different approaches suppressed AML cell growth at least in part by the induction of apoptosis.

In another study comparing the expression profiles of drug-resistant AML cells to drug-sensitive cells, an up-regulation of IGF-I could be associated with the resistance to the chemotherapeutical agent Ara-C (Abe et al., 2006). Consistently, IGF-I expression levels were found to be higher in patients at the refractory stage who received an Ara-C combined therapy, than in patients at diagnosis (Abe et al., 2006). It is a common finding that prolonged drug treatment can induce therapy resistance and that selected malignant cells can bypass the treatment efficacy by the activation of parallel signaling pathway. Indeed, sustained exposure of AML cells to tyrosine kinase inhibitors targeting FLT3 was shown to result in the activation of N-Ras and its signaling pathway (Piloto et al., 2007). Recently, another study described increased activation of the PI3K/Akt system upon treatment of AML patient blasts with the rapamycin derivative inhibitor everolimus (RAD001). Interestingly, the effect was explained by an IGF-I/IGF-IR autocrine signaling loop (Tamburini et al., 2007).

It is beyond question that we are still not familiar with all the sophisticated characteristics and reactions of cancer cells. However, current approaches targeting multiple signaling pathways definitively bear great potential in regard to treatment efficacy. In this notion, it was also our interest to investigate the potential of combining different targeting strategies. We could show that the use of NVP-AEW541 to block the IGF-IR kinase activity sensitized AML cells to chemotherapeutical agents and increased apoptosis. Besides, targeting the system further downstream by down-regulating or inhibiting the PI3K isoforms p110β or p110δ enhanced the action of etoposide or Ara-C. An important role of p110δ in AML cell proliferation was already described before (Billottet et al., 2006; Sujobert et al., 2005). However, we could show for the first time that the class I PI3K p110β was also widely expressed in the panel of AML cells analyzed and essentially contributed to cell growth.

While much attention has been paid to the understanding of the PI3K class I isoforms, little is known on the role of the other classes in regard to human cancer. Given the fact that also the class II isoforms were shown to integrate signals from growth factor receptors and to contribute to the generation of 3'-phosphoinositides, a potential role in cancer formation is certainly given (Arcaro et al., 2000). Indeed, recent studies have elucidated the role of PI3KC2β in cytoskeletal

organization, cell migration and survival of human tumor cells (Katso et al., 2006; Maffucci et al., 2005). In this context, we conducted an extensive analysis of PI3KC2β in a number of human tumors. We could show that PI3KC2β was widely expressed in a panel of AML, glioblastoma multiforme, medulloblastoma, neuroblastoma, and small cell lung cancer cells. Over-expression of the mRNA as well as the protein was visible in certain subgroups, going in hand with previous studies that showed up-regulation of PI3KC2β in a range of cancers including AML (Armstrong et al., 2002; Qian et al., 2002). By testing novel isoforms-specific inhibitors and siRNA to down-regulate PI3KC2β we could show that also this PI3K isoform bears great potential as a therapeutic target. Different cancer cell lines were highly sensitive to PI3KC2β inhibitors and strongly reduced cell proliferation rate in a dose-dependent manner. Besides, inhibiting PI3KC2β sensitized AML and glioblastoma cells to chemotherapeutical agents. Taken together, we could describe an important role of PI3KC2β for cell survival and chemo-sensitization in various tumors. Additionally, studying highly motile breast cancer cells underlined the finding that PI3KC2β essentially contributes to cancer cell motility.

Studying expression patterns of RTK signaling molecules in AML cells lead to the interesting finding of highly variable mTOR levels in the panel of cells analyzed. A significant contribution of mTOR to AML cell growth and cell cycle control had been described before and constitutive activation of signaling pathways involving mTOR was shown to commonly occur in AML patients (Kubota et al., 2004; Martelli et al., 2006; Recher et al., 2005). We therefore aimed at gaining a better understanding of this rather unexpected finding and compared cells expressing high levels of mTOR ($mTOR^{high}$ cells) to cells expressing low levels only ($mTOR^{low}$ cells). By first comparing growth factor-induced pathway activation we could show that $mTOR^{low}$ were not able to phosphorylate the mTOR downstream targets S6 protein and 4E-BP to the same extent as $mTOR^{high}$ cells. However, activation/phosphorylation of the upstream molecule Akt was comparable. In a next step we compared the response to the mTOR inhibitor rapamycin in the two groups of cells. Surprisingly, no striking differences in regard to cell proliferation, induction of apoptosis and cell cycle could be detected. This finding raised the question of additional targets of rapamycin in AML which was further underlined by the result that cells expression high levels of mTOR indeed seemed to be more dependent on this protein. siRNA-induced down-regulation of mTOR namely significantly reduced cell proliferation in $mTOR^{high}$ cells whereas

the growth rate of $mTOR^{low}$ was not affected. A siRNA screen was therefore aimed at uncovering human kinases which modulate the sensitivity to rapamycin in AML. First results screening $mTOR^{low}$ cells for proteins that had the strongest additive anti-proliferative effect brought the attention to certain RTKs such as the IGF-IR, FLT3 and the FGFR1, signal transducers such as SYK, ZAP70 and AKT1 to 3 as well as already well known oncogenes in leukemia like ABL1 and ABL2. Further experiments are now focused on validation of these candidates, on the one hand by the use of shRNA and on the other hand by the use of available inhibitors to specifically target the proteins. So far we could show that the candidates indeed play an important role in growth of the cancer cells and that targeting these kinases in combination with rapamycin bears great potential in regard to combinatorial cancer therapy. In a next step, $mTOR^{high}$ cells will be screened using the same kinase library. The comparison with the $mTOR^{low}$ cells will hopefully give further insight into the distinctness of the two groups and help elucidate the role of mTOR in AML cells.

In summary, the studies presented in this thesis underlined the significance of the RTK network in human cancer and yielded important findings which are relevant for the evaluation process of new molecular targets for AML.

5. REFERENCES

Abe, S., Funato, T., Takahashi, S., Yokoyama, H., Yamamoto, J., Tomiya, Y., Yamada-Fujiwara, M., Ishizawa, K., Kameoka, J., Kaku, M., Harigae, H. & Sasaki, T. (2006). Increased expression of insulin-like growth factor i is associated with Ara-C resistance in leukemia. *Tohoku J Exp Med*, **209,** 217-28.

Aggerholm, A., Gronbaek, K., Guldberg, P. & Hokland, P. (2000). Mutational analysis of the tumour suppressor gene MMAC1/PTEN in malignant myeloid disorders. *Eur J Haematol*, **65,** 109-13.

Ali, I.U., Schriml, L.M. & Dean, M. (1999). Mutational spectra of PTEN/MMAC1 gene: a tumor suppressor with lipid phosphatase activity. *J Natl Cancer Inst*, **91,** 1922-32.

Arcaro, A. & Wymann, M.P. (1993). Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *Biochem J*, **296 (Pt 2),** 297-301.

Arcaro, A., Zvelebil, M.J., Wallasch, C., Ullrich, A., Waterfield, M.D. & Domin, J. (2000). Class II phosphoinositide 3-kinases are downstream targets of activated polypeptide growth factor receptors. *Mol Cell Biol*, **20,** 3817-30.

Armstrong, S.A., Staunton, J.E., Silverman, L.B., Pieters, R., den Boer, M.L., Minden, M.D., Sallan, S.E., Lander, E.S., Golub, T.R. & Korsmeyer, S.J. (2002). MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. *Nat Genet*, **30,** 41-7.

Bartram, C.R., Ludwig, W.D., Hiddemann, W., Lyons, J., Buschle, M., Ritter, J., Harbott, J., Frohlich, A. & Janssen, J.W. (1989). Acute myeloid leukemia: analysis of ras gene mutations and clonality defined by polymorphic X-linked loci. *Leukemia*, **3,** 247-56.

Baxter, R.C. (1988). Characterization of the acid-labile subunit of the growth hormone-dependent insulin-like growth factor binding protein complex. *J Clin Endocrinol Metab*, **67,** 265-72.

Baxter, R.C. (2000). Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. *Am J Physiol Endocrinol Metab*, **278,** E967-76.

Beghini, A., Peterlongo, P., Ripamonti, C.B., Larizza, L., Cairoli, R., Morra, E. & Mecucci, C. (2000). C-kit mutations in core binding factor leukemias. *Blood*, **95,** 726-7.

Benistant, C., Chapuis, H. & Roche, S. (2000). A specific function for phosphatidylinositol 3-kinase alpha (p85alpha-p110alpha) in cell survival and for phosphatidylinositol 3-kinase beta (p85alpha-p110beta) in de novo DNA synthesis of human colon carcinoma cells. *Oncogene*, **19,** 5083-90.

Bi, L., Okabe, I., Bernard, D.J. & Nussbaum, R.L. (2002). Early embryonic lethality in mice deficient in the p110beta catalytic subunit of PI 3-kinase. *Mamm Genome*, **13,** 169-72.

Bi, L., Okabe, I., Bernard, D.J., Wynshaw-Boris, A. & Nussbaum, R.L. (1999). Proliferative defect and embryonic lethality in mice homozygous for a deletion in the p110alpha subunit of phosphoinositide 3-kinase. *J Biol Chem*, **274,** 10963-8.

Billottet, C., Grandage, V.L., Gale, R.E., Quattropani, A., Rommel, C., Vanhaesebroeck, B. & Khwaja, A. (2006). A selective inhibitor of the p110delta isoform of PI 3-kinase inhibits AML cell proliferation and survival and increases the cytotoxic effects of VP16. *Oncogene*, **25,** 6648-59.

Birkenkamp, K.U., Geugien, M., Lemmink, H.H., Kruijer, W. & Vellenga, E. (2001). Regulation of constitutive STAT5 phosphorylation in acute myeloid leukemia blasts. *Leukemia*, **15,** 1923-31.

Blakesley, V.A., Scrimgeour, A., Esposito, D. & Le Roith, D. (1996). Signaling via the insulin-like growth factor-I receptor: does it differ from insulin receptor signaling? *Cytokine Growth Factor Rev*, **7,** 153-9.

Brennan, F. & Foey, A. (2002). Cytokine regulation in RA synovial tissue: role of T cell/macrophage contact-dependent interactions. *Arthritis Res*, **4 Suppl 3,** S177-82.

Bromberg, J.F., Wrzeszczynska, M.H., Devgan, G., Zhao, Y., Pestell, R.G., Albanese, C. & Darnell, J.E., Jr. (1999). Stat3 as an oncogene. *Cell*, **98,** 295-303.

Brown, R.A., Domin, J., Arcaro, A., Waterfield, M.D. & Shepherd, P.R. (1999). Insulin activates the alpha isoform of class II phosphoinositide 3-kinase. *J Biol Chem*, **274,** 14529-32.

Cairoli, R., Beghini, A., Morello, E., Grillo, G., Montillo, M., Larizza, L. & Morra, E. (2005). Imatinib mesylate in the treatment of Core Binding Factor leukemias with KIT mutations. A report of three cases. *Leuk Res*, **29,** 397-400.

Cammenga, J., Horn, S., Bergholz, U., Sommer, G., Besmer, P., Fiedler, W. & Stocking, C. (2005). Extracellular KIT receptor mutants, commonly found in core binding factor AML, are constitutively active and respond to imatinib mesylate. *Blood*, **106,** 3958-61.

Camps, M., Ruckle, T., Ji, H., Ardissone, V., Rintelen, F., Shaw, J., Ferrandi, C., Chabert, C., Gillieron, C., Francon, B., Martin, T., Gretener, D., Perrin, D., Leroy, D., Vitte, P.A., Hirsch, E., Wymann, M.P., Cirillo, R., Schwarz, M.K. & Rommel, C. (2005). Blockade of PI3Kgamma suppresses joint inflammation and damage in mouse models of rheumatoid arthritis. *Nat Med*, **11,** 936-43.

Carboni, J.M., Lee, A.V., Hadsell, D.L., Rowley, B.R., Lee, F.Y., Bol, D.K., Camuso, A.E., Gottardis, M., Greer, A.F., Ho, C.P., Hurlburt, W., Li, A., Saulnier, M., Velaparthi, U., Wang, C., Wen, M.L., Westhouse, R.A., Wittman, M., Zimmermann, K., Rupnow, B.A. & Wong, T.W. (2005). Tumor development by transgenic expression of a constitutively active insulin-like growth factor I receptor. *Cancer Res*, **65,** 3781-7.

Care, R.S., Valk, P.J., Goodeve, A.C., Abu-Duhier, F.M., Geertsma-Kleinekoort, W.M., Wilson, G.A., Gari, M.A., Peake, I.R., Lowenberg, B. & Reilly, J.T. (2003). Incidence and prognosis of c-KIT and FLT3 mutations in core binding factor (CBF) acute myeloid leukaemias. *Br J Haematol*, **121,** 775-7.

Carow, C.E., Levenstein, M., Kaufmann, S.H., Chen, J., Amin, S., Rockwell, P., Witte, L., Borowitz, M.J., Civin, C.I. & Small, D. (1996). Expression of the hematopoietic growth factor receptor FLT3 (STK-1/Flk2) in human leukemias. *Blood*, **87,** 1089-96.

Chan, J.M., Stampfer, M.J., Ma, J., Gann, P., Gaziano, J.M., Pollak, M. & Giovannucci, E. (2002). Insulin-like growth factor-I (IGF-I) and IGF binding protein-3 as predictors of advanced-stage prostate cancer. *J Natl Cancer Inst*, **94,** 1099-106.

Chantry, D., Vojtek, A., Kashishian, A., Holtzman, D.A., Wood, C., Gray, P.W., Cooper, J.A. & Hoekstra, M.F. (1997). p110delta, a novel phosphatidylinositol 3-kinase catalytic subunit that associates with p85 and is expressed predominantly in leukocytes. *J Biol Chem*, **272,** 19236-41.

Cheong, J.W., Eom, J.I., Maeng, H.Y., Lee, S.T., Hahn, J.S., Ko, Y.W. & Min, Y.H. (2003). Phosphatase and tensin homologue phosphorylation in the C-terminal regulatory domain is frequently observed in acute myeloid leukaemia and associated with poor clinical outcome. *Br J Haematol*, **122,** 454-6.

Clayton, E., Bardi, G., Bell, S.E., Chantry, D., Downes, C.P., Gray, A., Humphries, L.A., Rawlings, D., Reynolds, H., Vigorito, E. & Turner, M. (2002). A crucial role for the p110delta subunit of phosphatidylinositol 3-kinase in B cell development and activation. *J Exp Med*, **196,** 753-63.

Cortes, J., Faderl, S., Estey, E., Kurzrock, R., Thomas, D., Beran, M., Garcia-Manero, G., Ferrajoli, A., Giles, F., Koller, C., O'Brien, S., Wright, J., Bai, S.A. & Kantarjian, H. (2005). Phase I study of BMS-214662, a farnesyl transferase inhibitor in patients with acute leukemias and high-risk myelodysplastic syndromes. *J Clin Oncol*, **23,** 2805-12.

Cortes, J., Giles, F., O'Brien, S., Thomas, D., Albitar, M., Rios, M.B., Talpaz, M., Garcia-Manero, G., Faderl, S., Letvak, L., Salvado, A. & Kantarjian, H. (2003). Results of imatinib mesylate therapy in patients with refractory or recurrent acute myeloid leukemia, high-risk myelodysplastic syndrome, and myeloproliferative disorders. *Cancer*, **97,** 2760-6.

Crackower, M.A., Oudit, G.Y., Kozieradzki, I., Sarao, R., Sun, H., Sasaki, T., Hirsch, E., Suzuki, A., Shioi, T., Irie-Sasaki, J., Sah, R., Cheng, H.Y., Rybin, V.O., Lembo, G., Fratta, L., Oliveira-dos-Santos, A.J., Benovic, J.L., Kahn, C.R., Izumo, S., Steinberg, S.F., Wymann, M.P., Backx, P.H. & Penninger, J.M. (2002). Regulation of myocardial contractility and cell size by distinct PI3K-PTEN signaling pathways. *Cell*, **110,** 737-49.

Crawley, J.B., Williams, L.M., Mander, T., Brennan, F.M. & Foxwell, B.M. (1996). Interleukin-10 stimulation of phosphatidylinositol 3-kinase and p70 S6 kinase is required for the proliferative but not the antiinflammatory effects of the cytokine. *J Biol Chem*, **271,** 16357-62.

Czauderna, F., Santel, A., Hinz, M., Fechtner, M., Durieux, B., Fisch, G., Leenders, F., Arnold, W., Giese, K., Klippel, A. & Kaufmann, J. (2003). Inducible shRNA expression for application in a prostate cancer mouse model. *Nucleic Acids Res*, **31,** e127.

Daughaday, W.H. & Rotwein, P. (1989). Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. *Endocr Rev*, **10,** 68-91.

De Meyts, P., Wallach, B., Christoffersen, C.T., Urso, B., Gronskov, K., Latus, L.J., Yakushiji, F., Ilondo, M.M. & Shymko, R.M. (1994). The insulin-like growth factor-I receptor. Structure, ligand-binding mechanism and signal transduction. *Horm Res*, **42,** 152-69.

Deangelo, D.J., Stone, R.M., Heaney, M.L., Nimer, S.D., Paquette, R.L., Klisovic, R.B., Caligiuri, M.A., Cooper, M.R., Lecerf, J.M., Karol, M.D., Sheng, S., Holford, N., Curtin, P.T., Druker, B.J. & Heinrich, M.C. (2006). Phase 1 clinical results with tandutinib (MLN518), a novel FLT3 antagonist, in patients with acute myelogenous leukemia or high-risk myelodysplastic syndrome: safety, pharmacokinetics, and pharmacodynamics. *Blood*, **108,** 3674-81.

Denley, A., Bonython, E.R., Booker, G.W., Cosgrove, L.J., Forbes, B.E., Ward, C.W. & Wallace, J.C. (2004). Structural determinants for high-affinity binding of insulin-like growth factor II to insulin receptor (IR)-A, the exon 11 minus isoform of the IR. *Mol Endocrinol*, **18,** 2502-12.

Deschler, B. & Lubbert, M. (2006). Acute myeloid leukemia: epidemiology and etiology. *Cancer*, **107,** 2099-107.

Downward, J. (2004). PI 3-kinase, Akt and cell survival. *Semin Cell Dev Biol*, **15,** 177-82.

Druker, B.J. (2003). Imatinib mesylate in the treatment of chronic myeloid leukaemia. *Expert Opin Pharmacother*, **4,** 963-71.

Estey, E. & Dohner, H. (2006). Acute myeloid leukaemia. *Lancet*, **368,** 1894-907.

Fang, G., Kim, C.N., Perkins, C.L., Ramadevi, N., Winton, E., Wittmann, S. & Bhalla, K.N. (2000). CGP57148B (STI-571) induces differentiation and apoptosis and sensitizes Bcr-Abl-positive human leukemia cells to apoptosis due to antileukemic drugs. *Blood*, **96,** 2246-53.

Farr, C.J., Saiki, R.K., Erlich, H.A., McCormick, F. & Marshall, C.J. (1988). Analysis of RAS gene mutations in acute myeloid leukemia by polymerase chain reaction and oligonucleotide probes. *Proc Natl Acad Sci U S A*, **85,** 1629-33.

Ferry, R.J., Jr., Cerri, R.W. & Cohen, P. (1999). Insulin-like growth factor binding proteins: new proteins, new functions. *Horm Res*, **51,** 53-67.

Fiedler, W., Mesters, R., Tinnefeld, H., Loges, S., Staib, P., Duhrsen, U., Flasshove, M., Ottmann, O.G., Jung, W., Cavalli, F., Kuse, R., Thomalla, J., Serve, H., O'Farrell, A.M., Jacobs, M., Brega, N.M., Scigalla, P., Hossfeld, D.K. & Berdel, W.E. (2003). A phase 2 clinical study of SU5416 in patients with refractory acute myeloid leukemia. *Blood*, **102,** 2763-7.

Fiedler, W., Serve, H., Dohner, H., Schwittay, M., Ottmann, O.G., O'Farrell, A.M., Bello, C.L., Allred, R., Manning, W.C., Cherrington, J.M., Louie, S.G., Hong, W., Brega, N.M., Massimini, G., Scigalla, P., Berdel, W.E. & Hossfeld, D.K. (2005). A phase 1 study of SU11248 in the treatment of patients with refractory or resistant acute myeloid leukemia (AML) or not amenable to conventional therapy for the disease. *Blood*, **105,** 986-93.

Fingar, D.C. & Blenis, J. (2004). Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. *Oncogene*, **23,** 3151-71.

Foey, A., Green, P., Foxwell, B., Feldmann, M. & Brennan, F. (2002). Cytokine-stimulated T cells induce macrophage IL-10 production dependent on phosphatidylinositol 3-kinase and p70S6K: implications for rheumatoid arthritis. *Arthritis Res*, **4,** 64-70.

Foukas, L.C., Claret, M., Pearce, W., Okkenhaug, K., Meek, S., Peskett, E., Sancho, S., Smith, A.J., Withers, D.J. & Vanhaesebroeck, B. (2006). Critical role for the p110alpha phosphoinositide-3-OH kinase in growth and metabolic regulation. *Nature*, **441,** 366-70.

Frohling, S., Scholl, C., Gilliland, D.G. & Levine, R.L. (2005). Genetics of myeloid malignancies: pathogenetic and clinical implications. *J Clin Oncol*, **23,** 6285-95.

Fruman, D.A., Mauvais-Jarvis, F., Pollard, D.A., Yballe, C.M., Brazil, D., Bronson, R.T., Kahn, C.R. & Cantley, L.C. (2000). Hypoglycaemia, liver necrosis and perinatal death in mice lacking all isoforms of phosphoinositide 3-kinase p85 alpha. *Nat Genet*, **26,** 379-82.

Fruman, D.A., Snapper, S.B., Yballe, C.M., Davidson, L., Yu, J.Y., Alt, F.W. & Cantley, L.C. (1999). Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85alpha. *Science*, **283,** 393-7.

Gari, M., Goodeve, A., Wilson, G., Winship, P., Langabeer, S., Linch, D., Vandenberghe, E., Peake, I. & Reilly, J. (1999). c-kit proto-oncogene exon 8 in-frame deletion plus insertion mutations in acute myeloid leukaemia. *Br J Haematol*, **105,** 894-900.

Gebauer, G., Jager, W. & Lang, N. (1998). mRNA expression of components of the insulin-like growth factor system in breast cancer cell lines, tissues, and metastatic breast cancer cells. *Anticancer Res*, **18,** 1191-5.

George, P., Bali, P., Cohen, P., Tao, J., Guo, F., Sigua, C., Vishvanath, A., Fiskus, W., Scuto, A., Annavarapu, S., Moscinski, L. & Bhalla, K. (2004). Cotreatment with 17-allylamino-demethoxygeldanamycin and FLT-3 kinase inhibitor PKC412 is highly effective against human acute myelogenous leukemia cells with mutant FLT-3. *Cancer Res*, **64,** 3645-52.

Giles, F.J., Stopeck, A.T., Silverman, L.R., Lancet, J.E., Cooper, M.A., Hannah, A.L., Cherrington, J.M., O'Farrell, A.M., Yuen, H.A., Louie, S.G., Hong, W., Cortes, J.E., Verstovsek, S., Albitar, M., O'Brien, S.M., Kantarjian, H.M. & Karp, J.E. (2003). SU5416, a small molecule tyrosine kinase receptor inhibitor, has biologic activity in patients with refractory acute myeloid leukemia or myelodysplastic syndromes. *Blood*, **102,** 795-801.

Gilliland, D.G. & Griffin, J.D. (2002). Role of FLT3 in leukemia. *Curr Opin Hematol*, **9,** 274-81.

Giovannucci, E., Pollak, M.N., Platz, E.A., Willett, W.C., Stampfer, M.J., Majeed, N., Colditz, G.A., Speizer, F.E. & Hankinson, S.E. (2000). A prospective study of plasma insulin-like growth factor-1 and binding protein-3 and risk of colorectal neoplasia in women. *Cancer Epidemiol Biomarkers Prev*, **9,** 345-9.

Grandage, V.L., Gale, R.E., Linch, D.C. & Khwaja, A. (2005). PI3-kinase/Akt is constitutively active in primary acute myeloid leukaemia cells and regulates survival and chemoresistance via NF-kappaB, Mapkinase and p53 pathways. *Leukemia*, **19,** 586-94.

Greenspan, F.S. & Gardner, D.G. (2001). Table of Normal Hormone Reference Ranges. In *Basic & Clinical Endocrinology* pp. 858t. McGraw-Hill Medical Publishing Division.

Griffin, J.D. (2001). Point mutations in the FLT3 gene in AML. *Blood*, **97,** 2193A-2193.

Guerreiro, A.S., Boller, D., Doepfner, K.T. & Arcaro, A. (2006). IGF-IR: potential role in antitumor agents. *Drug News Perspect*, **19,** 261-72.

Guo, Y.S., Jin, G.F., Townsend, C.M., Jr., Zhang, T., Sheng, H.M., Beauchamp, R.D. & Thompson, J.C. (1995). Insulin-like growth factor-II expression in carcinoma in colon cell lines: implications for autocrine actions. *J Am Coll Surg*, **181,** 145-54.

Gurbuxani, S., Vyas, P. & Crispino, J.D. (2004). Recent insights into the mechanisms of myeloid leukemogenesis in Down syndrome. *Blood*, **103,** 399-406.

Hahn, M., Li, W., Yu, C., Rahmani, M., Dent, P. & Grant, S. (2005). Rapamycin and UCN-01 synergistically induce apoptosis in human leukemia cells through a process that is regulated by the Raf-1/MEK/ERK, Akt, and JNK signal transduction pathways. *Mol Cancer Ther*, **4,** 457-70.

Hankinson, S.E., Willett, W.C., Colditz, G.A., Hunter, D.J., Michaud, D.S., Deroo, B., Rosner, B., Speizer, F.E. & Pollak, M. (1998). Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. *Lancet*, **351,** 1393-6.

Harman, S.M., Metter, E.J., Blackman, M.R., Landis, P.K. & Carter, H.B. (2000). Serum levels of insulin-like growth factor I (IGF-I), IGF-II, IGF-binding protein-3, and prostate-specific antigen as predictors of clinical prostate cancer. *J Clin Endocrinol Metab*, **85,** 4258-65.

Hayakawa, F., Towatari, M., Kiyoi, H., Tanimoto, M., Kitamura, T., Saito, H. & Naoe, T. (2000). Tandem-duplicated Flt3 constitutively activates STAT5 and MAP kinase and introduces autonomous cell growth in IL-3-dependent cell lines. *Oncogene*, **19,** 624-31.

Heinrich, M.C., Griffith, D.J., Druker, B.J., Wait, C.L., Ott, K.A. & Zigler, A.J. (2000). Inhibition of c-kit receptor tyrosine kinase activity by STI 571, a selective tyrosine kinase inhibitor. *Blood*, **96,** 925-32.

Hidalgo, M. & Rowinsky, E.K. (2000). The rapamycin-sensitive signal transduction pathway as a target for cancer therapy. *Oncogene*, **19,** 6680-6.

Hiddemann, W., Spiekermann, K., Buske, C., Feuring-Buske, M., Braess, J., Haferlach, T., Schoch, C., Kern, W., Schnittger, S., Berdel, W., Wormann, B., Heinecke, A., Sauerland, C. & Buchner, T. (2005). Towards a pathogenesis-oriented therapy of acute myeloid leukemia. *Crit Rev Oncol Hematol*, **56,** 235-45.

Hirsch, E., Katanaev, V.L., Garlanda, C., Azzolino, O., Pirola, L., Silengo, L., Sozzani, S., Mantovani, A., Altruda, F. & Wymann, M.P. (2000). Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science*, **287,** 1049-53.

Hizuka, N., Sukegawa, I., Takano, K., Asakawa, K., Horikawa, R., Tsushima, T. & Shizume, K. (1987). Characterization of insulin-like growth factor I receptors on human erythroleukemia cell line (K-562 cells). *Endocrinol Jpn*, **34,** 81-8.

Hubbard, S.R., Mohammadi, M. & Schlessinger, J. (1998). Autoregulatory mechanisms in protein-tyrosine kinases. *J Biol Chem*, **273,** 11987-90.

Hubbard, S.R. & Till, J.H. (2000). Protein tyrosine kinase structure and function. *Annu Rev Biochem*, **69,** 373-98.

Hunter, T. (1998). The Croonian Lecture 1997. The phosphorylation of proteins on tyrosine: its role in cell growth and disease. *Philos Trans R Soc Lond B Biol Sci*, **353,** 583-605.

Hunter, T. & Sefton, B.M. (1980). Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc Natl Acad Sci U S A*, **77,** 1311-5.

Ito, K., Tominaga, K., Suzuki, T., Jinnai, I. & Bessho, M. (2005). Successful treatment with imatinib mesylate in a case of minor BCR-ABL-positive acute myelogenous leukemia. *Int J Hematol*, **81,** 242-5.

James, J.A., Smith, M.A., Court, E.L., Yip, C., Ching, Y., Willson, C. & Smith, J.G. (2003). An investigation of the effects of the MEK inhibitor U0126 on apoptosis in acute leukemia. *Hematol J*, **4,** 427-32.

Jarrett, R.J. (1989). Cardiovascular disease and hypertension in diabetes mellitus. *Diabetes Metab Rev*, **5,** 547-58.

Jentsch-Ullrich, K., Pelz, A.F., Braun, H., Koenigsmann, M., Mohren, M., Wieacker, P. & Franke, A. (2004). Complete molecular remission in a patient with Philadelphia-chromosome positive acute myeloid leukemia after conventional therapy and imatinib. *Haematologica*, **89,** ECR15.

Jiang, G. & Hunter, T. (1999). Receptor signaling: when dimerization is not enough. *Curr Biol*, **9,** R568-71.

Jones, J.I. & Clemmons, D.R. (1995). Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev*, **16,** 3-34.

Jou, S.T., Carpino, N., Takahashi, Y., Piekorz, R., Chao, J.R., Carpino, N., Wang, D. & Ihle, J.N. (2002). Essential, nonredundant role for the phosphoinositide 3-kinase p110delta in signaling by the B-cell receptor complex. *Mol Cell Biol*, **22,** 8580-91.

Kaaks, R., Toniolo, P., Akhmedkhanov, A., Lukanova, A., Biessy, C., Dechaud, H., Rinaldi, S., Zeleniuch-Jacquotte, A., Shore, R.E. & Riboli, E. (2000). Serum C-peptide, insulin-like growth factor (IGF)-I, IGF-binding proteins, and colorectal cancer risk in women. *J Natl Cancer Inst*, **92,** 1592-600.

Kaleko, M., Rutter, W.J. & Miller, A.D. (1990). Overexpression of the human insulinlike growth factor I receptor promotes ligand-dependent neoplastic transformation. *Mol Cell Biol*, **10,** 464-73.

Kanakura, Y., Ikeda, H., Kitayama, H., Sugahara, H. & Furitsu, T. (1993). Expression, function and activation of the proto-oncogene c-kit product in human leukemia cells. *Leuk Lymphoma*, **10,** 35-41.

Kang, S., Bader, A.G. & Vogt, P.K. (2005). Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic. *Proc Natl Acad Sci U S A*, **102,** 802-7.

Karp, J.E., Lancet, J.E., Kaufmann, S.H., End, D.W., Wright, J.J., Bol, K., Horak, I., Tidwell, M.L., Liesveld, J., Kottke, T.J., Ange, D., Buddharaju, L., Gojo, I., Highsmith, W.E., Belly, R.T., Hohl, R.J., Rybak, M.E., Thibault, A. & Rosenblatt, J. (2001). Clinical and biologic activity of the

farnesyltransferase inhibitor R115777 in adults with refractory and relapsed acute leukemias: a phase 1 clinical-laboratory correlative trial. *Blood,* **97,** 3361-9.

Kato, H., Faria, T.N., Stannard, B., Roberts, C.T., Jr. & LeRoith, D. (1993). Role of tyrosine kinase activity in signal transduction by the insulin-like growth factor-I (IGF-I) receptor. Characterization of kinase-deficient IGF-I receptors and the action of an IGF-I-mimetic antibody (alpha IR-3). *J Biol Chem*, **268,** 2655-61.

Katso, R., Okkenhaug, K., Ahmadi, K., White, S., Timms, J. & Waterfield, M.D. (2001). Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Annu Rev Cell Dev Biol*, **17,** 615-75.

Katso, R.M., Pardo, O.E., Palamidessi, A., Franz, C.M., Marinov, M., De Laurentiis, A., Downward, J., Scita, G., Ridley, A.J., Waterfield, M.D. & Arcaro, A. (2006). Phosphoinositide 3-Kinase C2beta regulates cytoskeletal organization and cell migration via Rac-dependent mechanisms. *Mol Biol Cell*, **17,** 3729-44.

Keinan-Boker, L., Bueno De Mesquita, H.B., Kaaks, R., Van Gils, C.H., Van Noord, P.A., Rinaldi, S., Riboli, E., Seidell, J.C., Grobbee, D.E. & Peeters, P.H. (2003). Circulating levels of insulin-like growth factor I, its binding proteins -1,-2, -3, C-peptide and risk of postmenopausal breast cancer. *Int J Cancer*, **106,** 90-5.

Kelly, L.M. & Gilliland, D.G. (2002). Genetics of myeloid leukemias. *Annu Rev Genomics Hum Genet*, **3,** 179-98.

Kelly, L.M., Yu, J.C., Boulton, C.L., Apatira, M., Li, J., Sullivan, C.M., Williams, I., Amaral, S.M., Curley, D.P., Duclos, N., Neuberg, D., Scarborough, R.M., Pandey, A., Hollenbach, S., Abe, K., Lokker, N.A., Gilliland, D.G. & Giese, N.A. (2002). CT53518, a novel selective FLT3 antagonist for the treatment of acute myelogenous leukemia (AML). *Cancer Cell*, **1,** 421-32.

Khandwala, H.M., McCutcheon, I.E., Flyvbjerg, A. & Friend, K.E. (2000). The effects of insulin-like growth factors on tumorigenesis and neoplastic growth. *Endocr Rev*, **21,** 215-44.

Kim, S.C., Hahn, J.S., Min, Y.H., Yoo, N.C., Ko, Y.W. & Lee, W.J. (1999). Constitutive activation of extracellular signal-regulated kinase in human acute leukemias: combined role of activation of MEK, hyperexpression of extracellular signal-regulated kinase, and downregulation of a phosphatase, PAC1. *Blood*, **93,** 3893-9.

Kindler, T., Breitenbuecher, F., Marx, A., Beck, J., Hess, G., Weinkauf, B., Duyster, J., Peschel, C., Kirkpatrick, C.J., Theobald, M., Gschaidmeier, H., Huber, C. & Fischer, T. (2004). Efficacy and safety of imatinib in adult patients with c-kit-positive acute myeloid leukemia. *Blood*, **103,** 3644-54.

Kindler, T., Breitenbuecher, F., Marx, A., Hess, G., Gschaidmeier, H., Gamm, H., Kirkpatrick, C.J., Huber, C. & Fischer, T. (2003). Sustained complete hematologic remission after administration of the tyrosine kinase inhibitor imatinib mesylate in a patient with refractory, secondary AML. *Blood*, **101,** 2960-2.

Knapper, S., Burnett, A.K., Littlewood, T., Kell, W.J., Agrawal, S., Chopra, R., Clark, R., Levis, M.J. & Small, D. (2006a). A phase 2 trial of the FLT3 inhibitor lestaurtinib (CEP701) as first-line treatment for older patients with acute myeloid leukemia not considered fit for intensive chemotherapy. *Blood*, **108,** 3262-70.

Knapper, S., Mills, K.I., Gilkes, A.F., Austin, S.J., Walsh, V. & Burnett, A.K. (2006b). The effects of lestaurtinib (CEP701) and PKC412 on primary AML blasts: the induction of cytotoxicity varies with dependence on FLT3 signaling in both FLT3-mutated and wild-type cases. *Blood*, **108,** 3494-503.

Knight, Z.A., Gonzalez, B., Feldman, M.E., Zunder, E.R., Goldenberg, D.D., Williams, O., Loewith, R., Stokoe, D., Balla, A., Toth, B., Balla, T., Weiss, W.A., Williams, R.L. & Shokat, K.M. (2006). A pharmacological map of the PI3-K family defines a role for p110alpha in insulin signaling. *Cell*, **125,** 733-47.

Kolitz, J.E. (2006). Current therapeutic strategies for acute myeloid leukaemia. *Br J Haematol*, **134,** 555-72.

Kornfeld, S. (1992). Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors. *Annu Rev Biochem*, **61,** 307-30.

Korycka, A., Smolewski, P. & Robak, T. (2004). The influence of farnesyl protein transferase inhibitor R115777 (Zarnestra) alone and in combination with purine nucleoside analogs on acute myeloid leukemia progenitors in vitro. *Eur J Haematol*, **73,** 418-26.

Krajcik, R.A., Borofsky, N.D., Massardo, S. & Orentreich, N. (2002). Insulin-like growth factor I (IGF-I), IGF-binding proteins, and breast cancer. *Cancer Epidemiol Biomarkers Prev*, **11,** 1566-73.

Krugmann, S., Hawkins, P.T., Pryer, N. & Braselmann, S. (1999). Characterizing the interactions between the two subunits of the p101/p110gamma phosphoinositide 3-kinase and their role in the activation of this enzyme by G beta gamma subunits. *J Biol Chem*, **274,** 17152-8.

Kubota, Y., Ohnishi, H., Kitanaka, A., Ishida, T. & Tanaka, T. (2004). Constitutive activation of PI3K is involved in the spontaneous proliferation of primary acute myeloid leukemia cells: direct evidence of PI3K activation. *Leukemia*, **18,** 1438-40.

Kuriu, A., Ikeda, H., Kanakura, Y., Griffin, J.D., Druker, B., Yagura, H., Kitayama, H., Ishikawa, J., Nishiura, T., Kanayama, Y. & et al. (1991). Proliferation of human myeloid leukemia cell line associated with the tyrosine-phosphorylation and activation of the proto-oncogene c-kit product. *Blood*, **78,** 2834-40.

Kurkjian, C., Patel, S., Kamble, R., Dunn, S.T., Kern, W. & Kharfan-Dabaja, M.A. (2006). Acute promyelocytic leukemia and constitutional trisomy 21. *Cancer Genet Cytogenet*, **165,** 176-9.

Lacey, J.V., Jr., Hsing, A.W., Fillmore, C.M., Hoffman, S., Helzlsouer, K.J. & Comstock, G.W. (2001). Null association between insulin-like growth factors, insulin-like growth factor-binding proteins, and prostate cancer in a prospective study. *Cancer Epidemiol Biomarkers Prev*, **10,** 1101-2.

Lacronique, V., Boureux, A., Valle, V.D., Poirel, H., Quang, C.T., Mauchauffe, M., Berthou, C., Lessard, M., Berger, R., Ghysdael, J. & Bernard, O.A. (1997). A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. *Science*, **278,** 1309-12.

Laffargue, M., Calvez, R., Finan, P., Trifilieff, A., Barbier, M., Altruda, F., Hirsch, E. & Wymann, M.P. (2002). Phosphoinositide 3-kinase gamma is an essential amplifier of mast cell function. *Immunity*, **16,** 441-51.

Lancet, J.E., Gojo, I., Gotlib, J., Feldman, E.J., Greer, J., Liesveld, J.L., Bruzek, L.M., Morris, L., Park, Y., Adjei, A.A., Kaufmann, S.H., Garrett-Mayer, E., Greenberg, P.L., Wright, J.J. & Karp, J.E. (2006). A phase II study of the farnesyltransferase inhibitor tipifarnib in poor-risk and elderly patients with previously untreated acute myelogenous leukemia. *Blood*.

Lee, J. & Pilch, P.F. (1994). The insulin receptor: structure, function, and signaling. *Am J Physiol*, **266,** C319-34.

Lee, K.S., Lee, H.K., Hayflick, J.S., Lee, Y.C. & Puri, K.D. (2006a). Inhibition of phosphoinositide 3-kinase delta attenuates allergic airway inflammation and hyperresponsiveness in murine asthma model. *Faseb J*, **20,** 455-65.

Lee, K.S., Park, S.J., Kim, S.R., Min, K.H., Jin, S.M., Puri, K.D. & Lee, Y.C. (2006b). Phosphoinositide 3-kinase-delta inhibitor reduces vascular permeability in a murine model of asthma. *J Allergy Clin Immunol*, **118,** 403-9.

Lelievre, E., Bourbon, P.M., Duan, L.J., Nussbaum, R.L. & Fong, G.H. (2005). Deficiency in the p110alpha subunit of PI3K results in diminished Tie2 expression and Tie2(-/-)-like vascular defects in mice. *Blood*, **105,** 3935-8.

LeRoith, D., Kavsan, V.M., Koval, A.P. & Roberts, C.T., Jr. (1993). Phylogeny of the insulin-like growth factors (IGFs) and receptors: a molecular approach. *Mol Reprod Dev*, **35,** 332-6; discussion 337-8.

Levis, M., Allebach, J., Tse, K.F., Zheng, R., Baldwin, B.R., Smith, B.D., Jones-Bolin, S., Ruggeri, B., Dionne, C. & Small, D. (2002). A FLT3-targeted tyrosine kinase inhibitor is cytotoxic to leukemia cells in vitro and in vivo. *Blood*, **99,** 3885-91.

Li, Z., Jiang, H., Xie, W., Zhang, Z., Smrcka, A.V. & Wu, D. (2000). Roles of PLC-beta2 and -beta3 and PI3Kgamma in chemoattractant-mediated signal transduction. *Science*, **287,** 1046-9.

Liang, J. & Slingerland, J.M. (2003). Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. *Cell Cycle*, **2,** 339-45.

London, S.J., Yuan, J.M., Travlos, G.S., Gao, Y.T., Wilson, R.E., Ross, R.K. & Yu, M.C. (2002). Insulin-like growth factor I, IGF-binding protein 3, and lung cancer risk in a prospective study of men in China. *J Natl Cancer Inst*, **94,** 749-54.

Lukanova, A., Toniolo, P., Akhmedkhanov, A., Biessy, C., Haley, N.J., Shore, R.E., Riboli, E., Rinaldi, S. & Kaaks, R. (2001). A prospective study of insulin-like growth factor-I, IGF-binding proteins-1, -2 and -3 and lung cancer risk in women. *Int J Cancer*, **92,** 888-92.

Lunghi, P., Tabilio, A., Dall'Aglio, P.P., Ridolo, E., Carlo-Stella, C., Pelicci, P.G. & Bonati, A. (2003). Downmodulation of ERK activity inhibits the proliferation and induces the apoptosis of primary acute myelogenous leukemia blasts. *Leukemia*, **17,** 1783-93.

Luo, J., Sobkiw, C.L., Hirshman, M.F., Logsdon, M.N., Li, T.Q., Goodyear, L.J. & Cantley, L.C. (2006). Loss of class IA PI3K signaling in muscle leads to impaired muscle growth, insulin response, and hyperlipidemia. *Cell Metab*, **3,** 355-66.

Lyman, S.D. & Williams, D.E. (1995). Biology and potential clinical applications of flt3 ligand. *Curr Opin Hematol*, **2,** 177-81.

Ma, J., Pollak, M.N., Giovannucci, E., Chan, J.M., Tao, Y., Hennekens, C.H. & Stampfer, M.J. (1999). Prospective study of colorectal cancer risk in men and plasma levels of insulin-like growth factor (IGF)-I and IGF-binding protein-3. *J Natl Cancer Inst*, **91,** 620-5.

Maehama, T. & Dixon, J.E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem*, **273,** 13375-8.

Maffucci, T., Cooke, F.T., Foster, F.M., Traer, C.J., Fry, M.J. & Falasca, M. (2005). Class II phosphoinositide 3-kinase defines a novel signaling pathway in cell migration. *J Cell Biol*, **169,** 789-99.

Magalhaes, I.Q., Splendore, A., Emerenciano, M., Figueiredo, A., Ferrari, I. & Pombo-de-Oliveira, M.S. (2006). GATA1 mutations in acute leukemia in children with Down syndrome. *Cancer Genet Cytogenet*, **166,** 112-6.

Martelli, A.M., Nyakern, M., Tabellini, G., Bortul, R., Tazzari, P.L., Evangelisti, C. & Cocco, L. (2006). Phosphoinositide 3-kinase/Akt signaling pathway and its therapeutical implications for human acute myeloid leukemia. *Leukemia*, **20,** 911-28.

Mesters, R.M., Padro, T., Bieker, R., Steins, M., Kreuter, M., Goner, M., Kelsey, S., Scigalla, P., Fiedler, W., Buchner, T. & Berdel, W.E. (2001). Stable remission after administration of the receptor tyrosine kinase inhibitor SU5416 in a patient with refractory acute myeloid leukemia. *Blood*, **98,** 241-3.

Milella, M., Estrov, Z., Kornblau, S.M., Carter, B.Z., Konopleva, M., Tari, A., Schober, W.D., Harris, D., Leysath, C.E., Lopez-Berestein, G., Huang, Z. & Andreeff, M. (2002). Synergistic induction of apoptosis by simultaneous disruption of the Bcl-2 and MEK/MAPK pathways in acute myelogenous leukemia. *Blood*, **99,** 3461-4.

Min, Y.H., Eom, J.I., Cheong, J.W., Maeng, H.O., Kim, J.Y., Jeung, H.K., Lee, S.T., Lee, M.H., Hahn, J.S. & Ko, Y.W. (2003). Constitutive phosphorylation of Akt/PKB protein in acute myeloid leukemia: its significance as a prognostic variable. *Leukemia*, **17,** 995-7.

Mitsiades, C.S., Mitsiades, N.S., McMullan, C.J., Poulaki, V., Shringarpure, R., Akiyama, M., Hideshima, T., Chauhan, D., Joseph, M., Libermann, T.A., Garcia-Echeverria, C., Pearson, M.A., Hofmann, F., Anderson, K.C. & Kung, A.L. (2004). Inhibition of the insulin-like growth factor receptor-1 tyrosine kinase activity as a therapeutic strategy for multiple myeloma, other hematologic malignancies, and solid tumors. *Cancer Cell*, **5,** 221-30.

Mohi, M.G., Boulton, C., Gu, T.L., Sternberg, D.W., Neuberg, D., Griffin, J.D., Gilliland, D.G. & Neel, B.G. (2004). Combination of rapamycin and protein tyrosine kinase (PTK) inhibitors for the treatment of leukemias caused by oncogenic PTKs. *Proc Natl Acad Sci U S A*, **101,** 3130-5.

Murakami, M.S. & Rosen, O.M. (1991). The role of insulin receptor autophosphorylation in signal transduction. *J Biol Chem*, **266,** 22653-60.

Myers, M.G., Jr., Sun, X.J., Cheatham, B., Jachna, B.R., Glasheen, E.M., Backer, J.M. & White, M.F. (1993). IRS-1 is a common element in insulin and insulin-like growth factor-I signaling to the phosphatidylinositol 3'-kinase. *Endocrinology*, **132,** 1421-30.

Nakanishi, Y., Mulshine, J.L., Kasprzyk, P.G., Natale, R.B., Maneckjee, R., Avis, I., Treston, A.M., Gazdar, A.F., Minna, J.D. & Cuttitta, F. (1988). Insulin-like growth factor-I can mediate autocrine proliferation of human small cell lung cancer cell lines in vitro. *J Clin Invest*, **82,** 354-9.

Nakao, M., Yokota, S., Iwai, T., Kaneko, H., Horiike, S., Kashima, K., Sonoda, Y., Fujimoto, T. & Misawa, S. (1996). Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. *Leukemia*, **10,** 1911-8.

Neri, L.M., Borgatti, P., Tazzari, P.L., Bortul, R., Cappellini, A., Tabellini, G., Bellacosa, A., Capitani, S. & Martelli, A.M. (2003). The phosphoinositide 3-kinase/AKT1 pathway involvement in drug and all-trans-retinoic acid resistance of leukemia cells. *Mol Cancer Res*, **1,** 234-46.

Nielsen, T.O., Andrews, H.N., Cheang, M., Kucab, J.E., Hsu, F.D., Ragaz, J., Gilks, C.B., Makretsov, N., Bajdik, C.D., Brookes, C., Neckers, L.M., Evdokimova, V., Huntsman, D.G. & Dunn, S.E. (2004). Expression of the insulin-like growth factor I receptor and urokinase plasminogen activator in breast cancer is associated with poor survival: potential for intervention with 17-allylamino geldanamycin. *Cancer Res*, **64,** 286-91.

Ning, Z.Q., Li, J. & Arceci, R.J. (2001). Signal transducer and activator of transcription 3 activation is required for Asp(816) mutant c-Kit-mediated cytokine-independent survival and proliferation in human leukemia cells. *Blood*, **97,** 3559-67.

O'Farrell, A.M., Abrams, T.J., Yuen, H.A., Ngai, T.J., Louie, S.G., Yee, K.W., Wong, L.M., Hong, W., Lee, L.B., Town, A., Smolich, B.D., Manning, W.C., Murray, L.J., Heinrich, M.C. & Cherrington, J.M. (2003a). SU11248 is a novel FLT3 tyrosine kinase inhibitor with potent activity in vitro and in vivo. *Blood*, **101,** 3597-605.

O'Farrell, A.M., Foran, J.M., Fiedler, W., Serve, H., Paquette, R.L., Cooper, M.A., Yuen, H.A., Louie, S.G., Kim, H., Nicholas, S., Heinrich, M.C., Berdel, W.E., Bello, C., Jacobs, M., Scigalla, P., Manning, W.C., Kelsey, S. & Cherrington, J.M. (2003b). An innovative phase I clinical study demonstrates inhibition of FLT3 phosphorylation by SU11248 in acute myeloid leukemia patients. *Clin Cancer Res*, **9,** 5465-76.

O'Farrell, A.M., Yuen, H.A., Smolich, B., Hannah, A.L., Louie, S.G., Hong, W., Stopeck, A.T., Silverman, L.R., Lancet, J.E., Karp, J.E., Albitar, M., Cherrington, J.M. & Giles, F.J. (2004). Effects of SU5416, a small molecule tyrosine kinase receptor inhibitor, on FLT3 expression and phosphorylation in patients with refractory acute myeloid leukemia. *Leuk Res*, **28,** 679-89.

O'Gorman, D.M., McKenna, S.L., McGahon, A.J., Knox, K.A. & Cotter, T.G. (2000). Sensitisation of HL60 human leukaemic cells to cytotoxic drug-induced apoptosis by inhibition of PI3-kinase survival signals. *Leukemia*, **14,** 602-11.

Ohmura, E., Okada, M., Onoda, N., Kamiya, Y., Murakami, H., Tsushima, T. & Shizume, K. (1990). Insulin-like growth factor I and transforming growth factor alpha as autocrine growth factors in human pancreatic cancer cell growth. *Cancer Res*, **50,** 103-7.

Okkenhaug, K., Bilancio, A., Farjot, G., Priddle, H., Sancho, S., Peskett, E., Pearce, W., Meek, S.E., Salpekar, A., Waterfield, M.D., Smith, A.J. & Vanhaesebroeck, B. (2002). Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. *Science*, **297,** 1031-4.

Orme, S.M., McNally, R.J., Cartwright, R.A. & Belchetz, P.E. (1998). Mortality and cancer incidence in acromegaly: a retrospective cohort study. United Kingdom Acromegaly Study Group. *J Clin Endocrinol Metab*, **83,** 2730-4.

Osaki, M., Oshimura, M. & Ito, H. (2004). PI3K-Akt pathway: its functions and alterations in human cancer. *Apoptosis*, **9,** 667-76.

Padua, R.A., Guinn, B.A., Al-Sabah, A.I., Smith, M., Taylor, C., Pettersson, T., Ridge, S., Carter, G., White, D., Oscier, D., Chevret, S. & West, R. (1998). RAS, FMS and p53 mutations and poor clinical outcome in myelodysplasias: a 10-year follow-up. *Leukemia*, **12,** 887-92.

Palmqvist, R., Hallmans, G., Rinaldi, S., Biessy, C., Stenling, R., Riboli, E. & Kaaks, R. (2002). Plasma insulin-like growth factor 1, insulin-like growth factor binding protein 3, and risk of colorectal cancer: a prospective study in northern Sweden. *Gut*, **50,** 642-6.

Pandini, G., Frasca, F., Mineo, R., Sciacca, L., Vigneri, R. & Belfiore, A. (2002). Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved. *J Biol Chem*, **277,** 39684-95.

Patrucco, E., Notte, A., Barberis, L., Selvetella, G., Maffei, A., Brancaccio, M., Marengo, S., Russo, G., Azzolino, O., Rybalkin, S.D., Silengo, L., Altruda, F., Wetzker, R., Wymann, M.P., Lembo, G. & Hirsch, E. (2004). PI3Kgamma modulates the cardiac response to chronic pressure overload by distinct kinase-dependent and -independent effects. *Cell*, **118,** 375-87.

Patti, M.E. & Kahn, C.R. (1998). The insulin receptor--a critical link in glucose homeostasis and insulin action. *J Basic Clin Physiol Pharmacol*, **9,** 89-109.

Peeters, P., Raynaud, S.D., Cools, J., Wlodarska, I., Grosgeorge, J., Philip, P., Monpoux, F., Van Rompaey, L., Baens, M., Van den Berghe, H. & Marynen, P. (1997). Fusion of TEL, the ETS-variant gene 6 (ETV6), to the receptor-associated kinase JAK2 as a result of t(9;12) in a lymphoid and t(9;15;12) in a myeloid leukemia. *Blood*, **90,** 2535-40.

Pendergast, A.M., Quilliam, L.A., Cripe, L.D., Bassing, C.H., Dai, Z., Li, N., Batzer, A., Rabun, K.M., Der, C.J., Schlessinger, J. & et al. (1993). BCR-ABL-induced oncogenesis is mediated by direct interaction with the SH2 domain of the GRB-2 adaptor protein. *Cell*, **75,** 175-85.

Peruzzi, F., Prisco, M., Dews, M., Salomoni, P., Grassilli, E., Romano, G., Calabretta, B. & Baserga, R. (1999). Multiple signaling pathways of the insulin-like growth factor 1 receptor in protection from apoptosis. *Mol Cell Biol*, **19,** 7203-15.

Peters, G., Gongoll, S., Langner, C., Mengel, M., Piso, P., Klempnauer, J., Ruschoff, J., Kreipe, H. & von Wasielewski, R. (2003). IGF-1R, IGF-1 and IGF-2 expression as potential prognostic and predictive markers in colorectal-cancer. *Virchows Arch*, **443,** 139-45.

Piloto, O., Wright, M., Brown, P., Kim, K.T., Levis, M. & Small, D. (2007). Prolonged exposure to FLT3 inhibitors leads to resistance via activation of parallel signaling pathways. *Blood*, **109,** 1643-52.

Pollak, M.N., Schernhammer, E.S. & Hankinson, S.E. (2004). Insulin-like growth factors and neoplasia. *Nat Rev Cancer*, **4,** 505-18.

Pompetti, F., Spadano, A., Sau, A., Mennucci, A., Russo, R., Catinella, V., Franchi, P.G., Calabrese, G., Palka, G., Fioritoni, G. & Iacone, A. (2006). Long-term remission in BCR/ABL-positive AML-M6 patient treated with Imatinib Mesylate. *Leuk Res*.

Powis, G., Bonjouklian, R., Berggren, M.M., Gallegos, A., Abraham, R., Ashendel, C., Zalkow, L., Matter, W.F., Dodge, J., Grindey, G. & et al. (1994). Wortmannin, a potent and selective inhibitor of phosphatidylinositol-3-kinase. *Cancer Res*, **54,** 2419-23.

Qi, H., Xiao, L., Lingyun, W., Ying, T., Yi-Zhi, L., Shao-Xu, Y. & Quan, P. (2006). Expression of type 1 insulin-like growth factor receptor in marrow nucleated cells in malignant hematological disorders: correlation with apoptosis. *Ann Hematol*, **85,** 95-101.

Qian, Z., Fernald, A.A., Godley, L.A., Larson, R.A. & Le Beau, M.M. (2002). Expression profiling of CD34+ hematopoietic stem/ progenitor cells reveals distinct subtypes of therapy-related acute myeloid leukemia. *Proc Natl Acad Sci U S A*, **99,** 14925-30.

Rambaldi, A., Wakamiya, N., Vellenga, E., Horiguchi, J., Warren, M.K., Kufe, D. & Griffin, J.D. (1988). Expression of the macrophage colony-stimulating factor and c-fms genes in human acute myeloblastic leukemia cells. *J Clin Invest*, **81,** 1030-5.

Recher, C., Beyne-Rauzy, O., Demur, C., Chicanne, G., Dos Santos, C., Mas, V.M., Benzaquen, D., Laurent, G., Huguet, F. & Payrastre, B. (2005). Antileukemic activity of rapamycin in acute myeloid leukemia. *Blood*, **105,** 2527-34.

Renehan, A.G., O'Dwyer, S.T. & Shalet, S.M. (1999). Re: Prospective study of colorectal cancer risk in men and plasma levels of insulin-like growth factor (IGF)-I and IGF-binding protein-3. *J Natl Cancer Inst*, **91,** 2051-2.

Renehan, A.G., Zwahlen, M., Minder, C., O'Dwyer, S.T., Shalet, S.M. & Egger, M. (2004). Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. *Lancet*, **363,** 1346-53.

Ridge, S.A., Worwood, M., Oscier, D., Jacobs, A. & Padua, R.A. (1990). FMS mutations in myelodysplastic, leukemic, and normal subjects. *Proc Natl Acad Sci U S A*, **87,** 1377-80.

Riedel, H., Dull, T.J., Honegger, A.M., Schlessinger, J. & Ullrich, A. (1989). Cytoplasmic domains determine signal specificity, cellular routing characteristics and influence ligand binding of epidermal growth factor and insulin receptors. *Embo J*, **8,** 2943-54.

Rosen, C.J. (1999). Serum insulin-like growth factors and insulin-like growth factor-binding proteins: clinical implications. *Clin Chem*, **45,** 1384-90.

Sachdev, D., Hartell, J.S., Lee, A.V., Zhang, X. & Yee, D. (2004). A dominant negative type I insulin-like growth factor receptor inhibits metastasis of human cancer cells. *J Biol Chem*, **279,** 5017-24.

Samuels, Y., Wang, Z., Bardelli, A., Silliman, N., Ptak, J., Szabo, S., Yan, H., Gazdar, A., Powell, S.M., Riggins, G.J., Willson, J.K., Markowitz, S., Kinzler, K.W., Vogelstein, B. & Velculescu, V.E. (2004). High frequency of mutations of the PIK3CA gene in human cancers. *Science*, **304,** 554.

Sasaki, T., Irie-Sasaki, J., Horie, Y., Bachmaier, K., Fata, J.E., Li, M., Suzuki, A., Bouchard, D., Ho, A., Redston, M., Gallinger, S., Khokha, R., Mak, T.W., Hawkins, P.T., Stephens, L., Scherer, S.W., Tsao, M. & Penninger, J.M. (2000a). Colorectal carcinomas in mice lacking the catalytic subunit of PI(3)Kgamma. *Nature*, **406,** 897-902.

Sasaki, T., Irie-Sasaki, J., Jones, R.G., Oliveira-dos-Santos, A.J., Stanford, W.L., Bolon, B., Wakeham, A., Itie, A., Bouchard, D., Kozieradzki, I., Joza, N., Mak, T.W., Ohashi, P.S., Suzuki, A. & Penninger, J.M. (2000b). Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. *Science*, **287,** 1040-6.

Scappini, B., Onida, F., Kantarjian, H.M., Dong, L., Verstovsek, S., Keating, M.J. & Beran, M. (2001). Effects of signal transduction inhibitor 571 in acute myelogenous leukemia cells. *Clin Cancer Res*, **7,** 3884-93.

Schaffhausen, B. (1995). SH2 domain structure and function. *Biochim Biophys Acta*, **1242,** 61-75.

Schittenhelm, M., Aichele, O., Krober, S.M., Brummendorf, T., Kanz, L. & Denzlinger, C. (2003). Complete remission of third recurrence of acute myeloid leukemia after treatment with imatinib (STI-571). *Leuk Lymphoma*, **44,** 1251-3.

Schlessinger, J. (1988). Signal transduction by allosteric receptor oligomerization. *Trends Biochem Sci*, **13,** 443-7.

Schreiber, S.L. (1991). Chemistry and biology of the immunophilins and their immunosuppressive ligands. *Science*, **251,** 283-7.

Schu, P.V., Takegawa, K., Fry, M.J., Stack, J.H., Waterfield, M.D. & Emr, S.D. (1993). Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting. *Science*, **260,** 88-91.

Sebti, S.M. & Hamilton, A.D. (2000). Farnesyltransferase and geranylgeranyltransferase I inhibitors and cancer therapy: lessons from mechanism and bench-to-bedside translational studies. *Oncogene*, **19,** 6584-93.

Seger, R. & Krebs, E.G. (1995). The MAPK signaling cascade. *Faseb J*, **9,** 726-35.

Sell, C., Dumenil, G., Deveaud, C., Miura, M., Coppola, D., DeAngelis, T., Rubin, R., Efstratiadis, A. & Baserga, R. (1994). Effect of a null mutation of the insulin-like growth factor I receptor gene on growth and transformation of mouse embryo fibroblasts. *Mol Cell Biol*, **14,** 3604-12.

Shayesteh, L., Lu, Y., Kuo, W.L., Baldocchi, R., Godfrey, T., Collins, C., Pinkel, D., Powell, B., Mills, G.B. & Gray, J.W. (1999). PIK3CA is implicated as an oncogene in ovarian cancer. *Nat Genet*, **21,** 99-102.

Shimon, I. & Shpilberg, O. (1995). The insulin-like growth factor system in regulation of normal and malignant hematopoiesis. *Leuk Res*, **19,** 233-40.

Shioi, T., Kang, P.M., Douglas, P.S., Hampe, J., Yballe, C.M., Lawitts, J., Cantley, L.C. & Izumo, S. (2000). The conserved phosphoinositide 3-kinase pathway determines heart size in mice. *Embo J*, **19,** 2537-48.

Sklar, C.A., Mertens, A.C., Mitby, P., Occhiogrosso, G., Qin, J., Heller, G., Yasui, Y. & Robison, L.L. (2002). Risk of disease recurrence and second neoplasms in survivors of childhood cancer treated with growth hormone: a report from the Childhood Cancer Survivor Study. *J Clin Endocrinol Metab*, **87,** 3136-41.

Smith, B.D., Levis, M., Beran, M., Giles, F., Kantarjian, H., Berg, K., Murphy, K.M., Dauses, T., Allebach, J. & Small, D. (2004). Single-agent CEP-701, a novel FLT3 inhibitor, shows biologic and clinical activity in patients with relapsed or refractory acute myeloid leukemia. *Blood*, **103,** 3669-76.

Smolich, B.D., Yuen, H.A., West, K.A., Giles, F.J., Albitar, M. & Cherrington, J.M. (2001). The antiangiogenic protein kinase inhibitors SU5416 and SU6668 inhibit the SCF receptor (c-kit) in a human myeloid leukemia cell line and in acute myeloid leukemia blasts. *Blood*, **97,** 1413-21.

Spitz, M.R., Barnett, M.J., Goodman, G.E., Thornquist, M.D., Wu, X. & Pollak, M. (2002). Serum insulin-like growth factor (IGF) and IGF-binding protein levels and risk of lung cancer: a case-control study nested in the beta-Carotene and Retinol Efficacy Trial Cohort. *Cancer Epidemiol Biomarkers Prev*, **11,** 1413-8.

Stattin, P., Bylund, A., Rinaldi, S., Biessy, C., Dechaud, H., Stenman, U.H., Egevad, L., Riboli, E., Hallmans, G. & Kaaks, R. (2000). Plasma insulin-like growth factor-I, insulin-like growth factor-binding proteins, and prostate cancer risk: a prospective study. *J Natl Cancer Inst*, **92,** 1910-7.

Steffen, B., Muller-Tidow, C., Schwable, J., Berdel, W.E. & Serve, H. (2005). The molecular pathogenesis of acute myeloid leukemia. *Crit Rev Oncol Hematol*, **56,** 195-221.

Stirewalt, D.L. & Radich, J.P. (2003). The role of FLT3 in haematopoietic malignancies. *Nat Rev Cancer*, **3,** 650-65.

Stone, R.M., DeAngelo, D.J., Klimek, V., Galinsky, I., Estey, E., Nimer, S.D., Grandin, W., Lebwohl, D., Wang, Y., Cohen, P., Fox, E.A., Neuberg, D., Clark, J., Gilliland, D.G. & Griffin, J.D. (2005). Patients with acute myeloid leukemia and an activating mutation in FLT3 respond to a small-molecule FLT3 tyrosine kinase inhibitor, PKC412. *Blood*, **105,** 54-60.

Suire, S., Coadwell, J., Ferguson, G.J., Davidson, K., Hawkins, P. & Stephens, L. (2005). p84, a new Gbetagamma-activated regulatory subunit of the type IB phosphoinositide 3-kinase p110gamma. *Curr Biol*, **15,** 566-70.

Sujobert, P., Bardet, V., Cornillet-Lefebvre, P., Hayflick, J.S., Prie, N., Verdier, F., Vanhaesebroeck, B., Muller, O., Pesce, F., Ifrah, N., Hunault-Berger, M., Berthou, C., Villemagne, B., Jourdan, E., Audhuy, B., Solary, E., Witz, B., Harousseau, J.L., Himberlin, C., Lamy, T., Lioure, B., Cahn, J.Y., Dreyfus, F., Mayeux, P., Lacombe, C. & Bouscary, D. (2005). Essential role for the p110delta isoform in phosphoinositide 3-kinase activation and cell proliferation in acute myeloid leukemia. *Blood*, **106,** 1063-6.

Sukegawa, I., Hizuka, N., Takano, K., Asakawa, K. & Shizume, K. (1987). Decrease in IGF-I binding sites on human promyelocytic leukemia cell line (HL-60) with differentiation. *Endocrinol Jpn*, **34,** 365-72.

Tamburini, J., Chapuis, N., Bardet, V., Park, S., Sujobert, P., Willems, L., Ifrah, N., Dreyfus, F., Mayeux, P., Lacombe, C. & Bouscary, D. (2007). mTORC1 inhibition activates PI3K/Akt by up-regulating IGF-1R signaling in acute myeloid leukemia: rational for therapeutic inhibition of both pathways. *Blood*.

Tenen, D.G. (2003). Disruption of differentiation in human cancer: AML shows the way. *Nat Rev Cancer*, **3,** 89-101.

Tong, F.K., Chow, S. & Hedley, D. (2006). Pharmacodynamic monitoring of BAY 43-9006 (Sorafenib) in phase I clinical trials involving solid tumor and AML/MDS patients, using flow cytometry to monitor activation of the ERK pathway in peripheral blood cells. *Cytometry B Clin Cytom*, **70,** 107-14.

Toniolo, P., Bruning, P.F., Akhmedkhanov, A., Bonfrer, J.M., Koenig, K.L., Lukanova, A., Shore, R.E. & Zeleniuch-Jacquotte, A. (2000). Serum insulin-like growth factor-I and breast cancer. *Int J Cancer*, **88,** 828-32.

Towatari, M., Iida, H., Tanimoto, M., Iwata, H., Hamaguchi, M. & Saito, H. (1997). Constitutive activation of mitogen-activated protein kinase pathway in acute leukemia cells. *Leukemia*, **11,** 479-84.

Ueki, K., Yballe, C.M., Brachmann, S.M., Vicent, D., Watt, J.M., Kahn, C.R. & Cantley, L.C. (2002). Increased insulin sensitivity in mice lacking p85beta subunit of phosphoinositide 3-kinase. *Proc Natl Acad Sci U S A*, **99,** 419-24.

Urso, B., Cope, D.L., Kalloo-Hosein, H.E., Hayward, A.C., Whitehead, J.P., O'Rahilly, S. & Siddle, K. (1999). Differences in signaling properties of the cytoplasmic domains of the insulin receptor and insulin-like growth factor receptor in 3T3-L1 adipocytes. *J Biol Chem*, **274,** 30864-73.

Vanhaesebroeck, B. & Alessi, D.R. (2000). The PI3K-PDK1 connection: more than just a road to PKB. *Biochem J*, **346 Pt 3,** 561-76.

Vanhaesebroeck, B., Ali, K., Bilancio, A., Geering, B. & Foukas, L.C. (2005). Signalling by PI3K isoforms: insights from gene-targeted mice. *Trends Biochem Sci*, **30,** 194-204.

Vanhaesebroeck, B., Leevers, S.J., Panayotou, G. & Waterfield, M.D. (1997a). Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem Sci*, **22,** 267-72.

Vanhaesebroeck, B., Welham, M.J., Kotani, K., Stein, R., Warne, P.H., Zvelebil, M.J., Higashi, K., Volinia, S., Downward, J. & Waterfield, M.D. (1997b). P110delta, a novel phosphoinositide 3-kinase in leukocytes. *Proc Natl Acad Sci U S A*, **94,** 4330-5.

Viniou, N.A., Vassilakopoulos, T.P., Giakoumi, X., Mantzouranis, M. & Pangalis, G.A. (2004). Ida-FLAG plus imatinib mesylate-induced molecular remission in a patient with chemoresistant Ph1+ acute myeloid leukemia. *Eur J Haematol*, **72,** 58-60.

Vivanco, I. & Sawyers, C.L. (2002). The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer*, **2,** 489-501.

Vlahos, C.J., Matter, W.F., Hui, K.Y. & Brown, R.F. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem*, **269,** 5241-8.

Wang, C., Curtis, J.E., Geissler, E.N., McCulloch, E.A. & Minden, M.D. (1989). The expression of the proto-oncogene C-kit in the blast cells of acute myeloblastic leukemia. *Leukemia*, **3,** 699-702.

Webb, S.M., Casanueva, F. & Wass, J.A. (2002). Oncological complications of excess GH in acromegaly. *Pituitary*, **5,** 21-5.

Wechsler, J., Greene, M., McDevitt, M.A., Anastasi, J., Karp, J.E., Le Beau, M.M. & Crispino, J.D. (2002). Acquired mutations in GATA1 in the megakaryoblastic leukemia of Down syndrome. *Nat Genet*, **32,** 148-52.

Weisberg, E., Boulton, C., Kelly, L.M., Manley, P., Fabbro, D., Meyer, T., Gilliland, D.G. & Griffin, J.D. (2002). Inhibition of mutant FLT3 receptors in leukemia cells by the small molecule tyrosine kinase inhibitor PKC412. *Cancer Cell*, **1,** 433-43.

Wolk, A., Mantzoros, C.S., Andersson, S.O., Bergstrom, R., Signorello, L.B., Lagiou, P., Adami, H.O. & Trichopoulos, D. (1998). Insulin-like growth factor 1 and prostate cancer risk: a population-based, case-control study. *J Natl Cancer Inst*, **90,** 911-5.

Wu, J., Wong, W.W., Khosravi, F., Minden, M.D. & Penn, L.Z. (2004). Blocking the Raf/MEK/ERK pathway sensitizes acute myelogenous leukemia cells to lovastatin-induced apoptosis. *Cancer Res*, **64,** 6461-8.

Wurmser, A.E., Gary, J.D. & Emr, S.D. (1999). Phosphoinositide 3-kinases and their FYVE domain-containing effectors as regulators of vacuolar/lysosomal membrane trafficking pathways. *J Biol Chem*, **274,** 9129-32.

Xu, Q., Simpson, S.E., Scialla, T.J., Bagg, A. & Carroll, M. (2003). Survival of acute myeloid leukemia cells requires PI3 kinase activation. *Blood*, **102,** 972-80.

Yamaguchi, M. & Konishi, I. (2003). [Successful treatment with imatinib mesylate for Philadelphia chromosome-positive refractory acute myeloid leukemia]. *Rinsho Ketsueki*, **44,** 254-6.

Yamamoto, K., Altschuler, D., Wood, E., Horlick, K., Jacobs, S. & Lapetina, E.G. (1992). Association of phosphorylated insulin-like growth factor-I receptor with the SH2 domains of phosphatidylinositol 3-kinase p85. *J Biol Chem*, **267,** 11337-43.

Yamamoto, Y., Kiyoi, H., Nakano, Y., Suzuki, R., Kodera, Y., Miyawaki, S., Asou, N., Kuriyama, K., Yagasaki, F., Shimazaki, C., Akiyama, H., Saito, K., Nishimura, M., Motoji, T., Shinagawa, K.,

Takeshita, A., Saito, H., Ueda, R., Ohno, R. & Naoe, T. (2001). Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. *Blood*, **97,** 2434-9.

Yasui, H., Hideshima, T., Richardson, P.G. & Anderson, K.C. (2006). Novel therapeutic strategies targeting growth factor signalling cascades in multiple myeloma. *Br J Haematol*, **132,** 385-97.

Yee, D., Paik, S., Lebovic, G.S., Marcus, R.R., Favoni, R.E., Cullen, K.J., Lippman, M.E. & Rosen, N. (1989). Analysis of insulin-like growth factor I gene expression in malignancy: evidence for a paracrine role in human breast cancer. *Mol Endocrinol*, **3,** 509-17.

Yee, K.W., O'Farrell, A.M., Smolich, B.D., Cherrington, J.M., McMahon, G., Wait, C.L., McGreevey, L.S., Griffith, D.J. & Heinrich, M.C. (2002). SU5416 and SU5614 inhibit kinase activity of wild-type and mutant FLT3 receptor tyrosine kinase. *Blood*, **100,** 2941-9.

Yousif, M.H., Benter, I.F., Hares, N., Canatan, H. & Akhtar, S. (2006). Phosphoinositide 3-kinase mediated signalling contributes to development of diabetes-induced abnormal vascular reactivity of rat carotid artery. *Cell Biochem Funct*, **24,** 13-22.

Yu, H., Spitz, M.R., Mistry, J., Gu, J., Hong, W.K. & Wu, X. (1999). Plasma levels of insulin-like growth factor-I and lung cancer risk: a case-control analysis. *J Natl Cancer Inst*, **91,** 151-6.

Zdychova, J. & Komers, R. (2005). Emerging role of Akt kinase/protein kinase B signaling in pathophysiology of diabetes and its complications. *Physiol Res*, **54,** 1-16.

Zeng, Z., Samudio, I.J., Zhang, W., Estrov, Z., Pelicano, H., Harris, D., Frolova, O., Hail, N., Jr., Chen, W., Kornblau, S.M., Huang, P., Lu, Y., Mills, G.B., Andreeff, M. & Konopleva, M. (2006). Simultaneous inhibition of PDK1/AKT and Fms-like tyrosine kinase 3 signaling by a small-molecule KP372-1 induces mitochondrial dysfunction and apoptosis in acute myelogenous leukemia. *Cancer Res*, **66,** 3737-46.

Zhao, S., Konopleva, M., Cabreira-Hansen, M., Xie, Z., Hu, W., Milella, M., Estrov, Z., Mills, G.B. & Andreeff, M. (2004). Inhibition of phosphatidylinositol 3-kinase dephosphorylates BAD and promotes apoptosis in myeloid leukemias. *Leukemia*, **18,** 267-75.

Zhou, M.M. & Fesik, S.W. (1995). Structure and function of the phosphotyrosine binding (PTB) domain. *Prog Biophys Mol Biol*, **64,** 221-35.

Zumkeller, W. & Burdach, S. (1999). The insulin-like growth factor system in normal and malignant hematopoietic cells. *Blood*, **94,** 3653-7.

6. CURRICULUM VITAE

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2004	Master Degree in Zoology, dipl. zool.
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Diet-dependent effects of the Drosophila Mnk1/Mnk2 homolog Lk6 on growth via eIF4E
Curr Biol. 2005 Jan 11;15(1):24-30.

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Drug News Perspect. 2006 Jun;19(5):261-72

2007

Doepfner KT, Boller D, De Laurentiis A, Guerreiro AS, Marinov M and Arcaro A: Recent *Patents of Gene Sequences Relative to the Phosphatidylinositol 3-kinase / Akt Pathway and their Relevance to Drug Discovery.*
Recent Patents on DNA & Gene Sequences 2007, 1, 9-23

Arcaro A, Doepfner KT, Boller D, Guerreiro AS, Shalaby T, Delattre O, Grotzer MA, and Fischer B
Autocrine Signaling by Insulin and the Insulin Receptor Controls Proliferation and Survival of Malignant Brain Tumor Cells
Biochem J. 2007 Aug 15;406(1):57-66

Doepfner KT, Spertini O, and Arcaro A
Autocrine insulin-like growth factor signaling promotes growth and survival of human acute myeloid leukemia cells via the Phosphatidylinositol-3 kinase pathway
Leukemia. 2007 Sep;21(9):1921-30. Epub 2007 Jun 21

Doepfner KT, Boller D, and Arcaro A
Targeting Receptor Tyrosine Kinase Signaling in Acute Myeloid Leukemia
Crit Rev Oncol Hematol. 2007 Sep;63(3):215-30. Epub 2007 Jul 19

Boller D, Schramm A, Doepfner KT, Shalaby T, Von Bueren OA, Eggert A, Grotzer MA, Arcaro A
Targeting the phosphoinositide 3-kinase isoform p110δ impairs growth and survival in neuroblastoma cells
Clinical Cancer Research (Manuscript in press)

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Posters / Oral presentations

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5th Day of Clinical Research, University Hospital Zurich (Poster)

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Journée des Jeunes Chercheurs en Pédiatrie, University of Bern (Oral Presentation)

Doepfner KT, Spertini O, Arcaro A
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Targeting the Kinome, Congress Center Basel (Poster)

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mTOR Expression Levels and Rapamycin Sensitivity in Acute Myeloid Leukemia Cells
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Doepfner KT, Spertini O, Downward J, Arcaro A
Investigation of the Molecular Determinants of Rapamycin Sensitivity in Acute Myeloid Leukemia Cells
BACR - Cell Signalling and Novel Cancer Therapeutics, London (Poster)

Boller D, Schramm A, Doepfner KT, Shalaby T, Von Bueren OA, Eggert A, Grotzer MA, Arcaro A
Targeting the phosphoinositide 3-kinase isoform p110δ impairs growth and survival in neuroblastoma cells
BACR - Cell Signalling and Novel Cancer Therapeutics, London (Poster)

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